An Investigation into the Recovery of Function after Different Types of Injury and Repair of Peripheral Nerve — A Comparison Between Non-diabetic and STZ Diabetic Rats.

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Abstract

1. Successful nerve regeneration depends on the type of injury, the method of repair and the metabolic status of the animal.

2. A state similar to poorly controlled type I diabetes mellitus in man was induced and maintained in rats using streptozotocin. Since this state is irreversible, these animals provided a suitable model for the study of nerve regeneration in diabetes over a period of 150 days.

3. Two methods of nerve injury (crush and transection) and 3 methods of repair (epineurial suture, nerve graft and muscle graft) were compared electrophysiologically and histologically, in diabetic and non-diabetic animals 150 days after operation.

4. It has been confirmed that the diabetic state causes a reduction in nerve conduction velocity.

5. Recovery of conduction velocity has been shown to be greater after nerve crush and nerve-to-nerve suture than after nerve graft and muscle graft, although the recovery after 150 days may have been incomplete. The recovery of conduction velocity after muscle grafting was not significantly poorer than after nerve grafting. The diabetic state did not affect the degree of recovery of nerve conduction velocity after nerve injury.

6. It was found that by 150 days, recovery to control values of axon and nerve fibre diameters was not attained. There was a gradation in recovery of fibre and axon diameter from: nerve crush > nerve-to-nerve suture > nerve graft > muscle graft.

7. Recovery of axon and fibre diameter was significantly poorer in the diabetic nerve crush group compared to that in the non-diabetic nerve crush group ($p > 0.01$). It has been proposed that this result was because of poorer regeneration in diabetic nerve.

8. It was found that the best recovery of peroneal cutaneous receptive field area was achieved after nerve crush and nerve-to-nerve suture and the worst after nerve grafting and muscle grafting. Recovery was poorer in the diabetic animals compared with like groups of non-diabetic animals, however, the difference was only significant after muscle grafting ($p > 0.01$).

9. Isometric tetanic and twitch tensions were induced in reinnervated soleus muscle to measure the recovery of motor function. Tensions of both types remained reduced 150 days after nerve injury and repair. Recovery was more complete after nerve crush than after injury of the neurotmesis type and subsequent repair. There was no difference in the recovery of soleus muscle twitch or tetanic tension in any of the diabetic groups of animals. The mean mass of soleus muscles removed from the diabetic animals was also less than that recorded from the non-diabetics. There was no significant difference in the recovery of twitch or tetanic tension between like groups of non-diabetic and diabetic animals. It seems that the recovery of motor function after nerve injury and repair is no worse in diabetic rats.

10. Using small amplitude vibration, it was demonstrated that a reduced reflex response is produced in muscles after nerve crush injury and a reflex response cannot be obtained from a muscle if the nerve to that muscle has been divided. This is probably owing to a combination of: a decreased number of functional reconnections being made and formation of inappropriate connections.

11. Doppler flowmetry indicated that the change in blood flow through the sciatic nerve after injury and repair, in response to methoxamine, did not return to normal. Blood flow was reduced in the sciatic nerve of rats in all operated groups but was poorest in the muscle graft groups of both non-diabetic and diabetic animals. A decreased blood flow could be a factor affecting recovery of function after nerve injury. A small decrease in blood flow (7%) was observed in the peripheral nerves of diabetic rats. This decrease is unlikely to be the only determinant of neuropathic change in peripheral nerve in STZ diabetic rats.

I, Agneta Carol Fullarton, declare that this thesis has been composed totally by myself and that the work reported here is my own work. Any technical assistance obtained has been acknowledged.

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CONTENTS

Abbreviations

AC alternating current

ADH anti-diuretic hormone

ATP adenosine triphosphate

C non-diabetic control group

Ca^{2+} calcium ions

ChAc choline acetylase

c.i. confidence interval

CoA coenzyme A

CNTF ciliary neurotrophic factor

CR non-diabetic nerve crush group

c.v. coefficient of variation

CV conduction velocity/ m s^{-1}

CV ratio ratio of the values of conduction velocity from left/operated and right/unoperated sciatic nerves

DAB diaminobenzidine tetrahydrochloride

DC direct current

DC diabetic control group

DCR diabetic crush group

d.f. degrees of freedom

DM diabetic muscle graft group

DNG diabetic nerve graft group

DNN diabetic nerve-to-nerve epineurial suture group

E.M.G. electromyogram

f frequency/ Hz

F tension or force/ N

GABA gamma-amino-butyric acid

η dynamic viscosity/ Pa s

H_2O water

IDDM insulin-dependent diabetes mellitus

IGF-I insulin-like growth factor-I

l length/ m

m mean of observations (sample mean)

MAC minimum alveolar concentration

MFLUX value of flux after adminiatration of methoxamine

MG non-diabetic muscle graft group

MI myoinositol

MNGF motor nerve growth factor

n sample size

Na^+/ K^+ ATP-ase sodium/ potassium adenosine triphosphatase

NAD^+ nicotine adenine dinucleotide

NADH reduced nicotine adenine dinucleotide

$NADP^+$ nicotine adenine dinucleotide phosphate

NADP reduced nicotine adenine dinucleotide phosphate

NG non-diabetic nerve graft group

NGF nerve growth factor

NIDDM non-insulin-dependent diabetes mellitus

NN non-diabetic nerve-to-nerve epineurial suture group

NS not significant

O_2 oxygen

ODC ornithine decarboxylase

OsO_4 osmium tetroxide

p probability

P pressure/ Pa

PI phosphatidylinositol

Q heat/ J

$\dot{Q}$ flow/ $m^3\ s^{-1}$

r radius/ m

$r_{1/2}$ time to half relaxation of muscle twitch/ s

RF peroneal cutaneous receptive field area/ cm^2

RF ratio ratio of the values of the cutaneous receptive field areas of the left and the right peroneal nerves

RICB resistance to ischaemic conduction block

rRNA ribosomal ribonucleic acid

$\underline{s}$ number of samples

s distance/ m

SCa slow component-a of axonal transport

SCb slow component-b of axonal transport

SD standard deviation of sample

SEM standard error of the mean

SND standard normal distribution

STD standard deviation of the population

STZ streptozotocin

t time to peak tension/ s

T period/ s

$t_{1/2}$ time to half peak tension/ s

TBS tris-buffered saline

t_d duration of muscle twitch/ s

TESPA 3-aminopropyltriethoxyxylane

T_d time at the end of the muscle twitch/ s

T_0 time at the beginning of the muscle twitch/ s

tRNA transfer ribonucleic acid

TT time-tension integral/ N s

TTI time-tension index/ N

u population mean

v velocity/ m s^{-1}

x,y variables

CHAPTER 1

General Introduction

1. General Introduction.

"Few physiologists..., now maintain the existence of
a nervous system in vegetables."
Carpenter, 1837.

Although the present day scientist knows significantly more about the anatomy and physiology of the peripheral nervous system, the surgeon knows that repair of a peripheral nerve rarely results in complete structural and functional recovery. Fortunately, with the advent of anaesthesia and improvements in surgical technique, the prognosis for a patient with peripheral nerve injury is not as bad as it once was. However, there is still much to be learned about the factors affecting nerve regeneration and the possible implications for improving the outcome after nerve surgery.

Much work has been done on the changes in numbers of axons, their size, and degree of myelination of regenerated peripheral nerve fibres. After nerve section and repair in rabbits, it was found that numbers of axons remained greater distal to the site of the nerve injury than proximal to the site of injury (Allt, 1976), although it is known that recovery of motor and sensory function is rarely complete after nerve transection injuries (Seckel, 1990). Gutmann and Sanders (1943) described two processes in the regeneration of nerve after injury which were: the outgrowth of axons from the distal stump, their arrival and connection with the periphery, and also the reconstitution of fibre numbers, diameters and "medullation". These authors found that maturation of regenerated nerve fibres after nerve transection and repair can take up to a year to be fully completed and nerve fibre diameters may never reach their original size, although it is known that

function is restored much sooner (Gutmann, Guttmann, Medawar and Young, 1942). Therefore, results from studies on the size, myelination or number of regenerated fibres after nerve injury and repair do not provide information about the degree of functional recovery that a patient can expect after nerve injury. Some of the aims of the work presented in this thesis were to investigate the differences in the recovery of function after different methods of nerve injury and repair. Other aims of the work presented here were to investigate nerve regeneration and recovery of function in experimental diabetes (see section 1.4). The possible implications of a less than optimal blood supply in nerve regeneration are dealt with in the work presented in chapter 8.

The recovery of function after different types of nerve injury and repair was investigated in the work presented in this thesis. Therefore, the purpose of the general introduction presented here is briefly to outline the process of peripheral nerve regeneration and the factors affecting it; with special attention to the type of nerve injury and to other aspects of nerve regeneration that were possibly influential in determining the outcome of the experiments presented in this thesis. The purpose of the second part of the general introduction is to outline the effects of diabetes mellitus on peripheral nerve and nerve regeneration. Current knowledge from research into the different aspects of recovery after nerve injury and repair, that were investigated in the work presented in this thesis, are discussed in the introductions of chapters 3 to 8.

1.1 Degeneration and Regeneration.

Changes in the neuronal cell bodies.

After nerve injury, the cell bodies of the damaged nerve fibres, in the dorsal root ganglia or the anterior horn of the spinal cord, undergo a

process called chromatolysis. The changes in the cell body include swelling, displacement of the nucleus and dispersion of Nissl substance (Seckel, 1990). Although the reactive changes in the neuronal cell bodies are poorly understood and some cells may not recover, changes in the cell body biochemistry include a switch from production of substances for neurotransmission to a production of material for axonal regeneration (Seckel, 1990). Kanje, Fransson, Edström and Löwkvist (1986) found that the activity of ornithine decarboxylase (ODC) in the dorsal root gangia of frogs was significantly increased 2 days after crushing the sciatic nerve and they stated that an increase in ODC activity appears to be necessary for the chromatolytic response of cell bodies. The aforementioned authors also found that inhibition of ODC activity prevented outgrowth of sensory axons. ODC is the rate-limiting enzyme in the polyamine biosynthetic pathway and polyamines are involved in nucleic acid and protein synthesis. Protein synthesized in the cell body is transported by slow axonal transport to the site of nerve injury. Slow axonal transport can be divided into two components: slow component-a (SCa) and slow component-b (SCb). Wujek and Lasek (1983) found that the rate of axonal regeneration in rats corresponded to the rate of axonal transport of SCb and they suggested that this component of axonal transport could be the rate-limiting step in axonal regeneration. Therefore, it seems possible that ODC activity could affect protein synthesis which subsequently affects the rate of axonal transport. Growth-associated proteins are also transported to the site of nerve injury but by fast axonal transport.

Traumatic degeneration.

Traumatic degeneration is the name given to the changes that take place in the nerve, proximal to the site of injury. Approximately 1 hour after

crush or transection of a peripheral nerve, there is retraction of myelin on either side of the nodes of Ranvier. Nodal gaps are increased in the nerve up to 4 cm proximal to the site of the nerve injury and Schmidt-Lanterman incisures become dilated. Seddon (1972) said that the extent of retrograde degeneration depends on the type of injury sustained. Where large amounts of "commotion" is produced, for example by gun-shot injury, retrograde changes can extend for centimetres proximal to the injury. A decrease in the size of axonal diameter has been reported to occur proximal to both nerve crush and nerve transection and suture injuries in rabbits, with a concommitant decrease in nerve conduction velocity (Cragg and Thomas, 1961). These authors found that the conduction velocities returned to normal by 200 days and axon diameters returned to almost normal by 225 days after both types of injury.

Wallerian degeneration.

Distal to the nerve injury, the process of degeneration is called Wallerian degeneration. During the acute phase of degeneration (the first 72 hours), the myelin sheath breaks down into 'ellipsoids' within which axonal material is degraded. The reason for the collapse of the nerve after injury may be because of a loss of axonal turgor pressure or alternatively, the collapse could be caused by the action of hydrolytic enzymes (Allt, 1976). After about 7 days, the ellipsoids are degraded further into spherical lipid droplets which are phagocytized further by Schwann cells and macrophages. These cells migrate into the region of axonal degeneration and account largely for the increase in nuclear population that is observed after nerve injury. Abercrombie and Johnson (1946) found that 25 days after injury to the sciatic nerve in rabbits, the nuclear population

was increased to 8 times the control values. The cells in the region of axonal degeneration also include endoneurial fibrocytes (Thomas, 1966).

It has been found that at the site of injury, there is a large influx of Ca^{2+} into the neurons which probably causes the disruption of neurofilaments and microtubules (Allt, 1976). The influx of Ca^{2+} may well be due to inhibition of those mechanisms whose function it is to exclude Ca^{2+} from the cell. It has been found that 24 to 48 hours after nerve damage, there is swelling of axonal mitochondria which then break up into fragments. Combined with a decrease in blood supply after nerve crush or section, the destruction of mitochondria could result in poor production and availability of high energy compounds which, in turn, affect the energy dependent mechanisms of the cell, including those that exclude Ca^{2+} from the cell.

Thomas (1964) observed that, after transection of the nerve to the medial head of the gastrocnemius muscle in the rabbit, the basement membranes that surround Schwann cells persisted and formed tubes within which Schwann cells proliferated. The Schwann tubes (or endoneurial sheath/tube) described by Thomas (1964) have also been called basement membrane or basal lamina tubes although strictly, the latter are not the same. The basal lamina is a synonym for the lamina densa which is just one of the three components of the basement membrane (lamina lucida, lamina densa and reticular lamina). The terms are often used interchangeably; in the work presented in this thesis, the terms basement membrane or endoneurial tube are used. Thomas (1966) observed that, after transection of the sural nerve in rats and rabbits, the columns of Schwann cells that extended into the nerve gap from the distal stump were surrounded by a common basement membrane. The cords of Schwann cells are known as the bands of Büngner. Thomas (1966) observed that the cords of Schwann cells were contained in a connective tissue framework consisting of

fibroblasts, blood vessels, leucocytes and macrophages. Mast cells have been observed in the region of the nerve gap their function may be to cause an increase in vascular permeability (Allt, 1976).

The initial breakdown products of axons after nerve transection cause the proliferation of Schwann cells which form the bands of Büngner. The Schwann cells remove axon and myelin debris. Subsequently, however, a regenerating axon is required for the differentiation of Schwann cells and production of myelin sheaths. Allt (1976) said that it has been found that Schwann cell migration and proliferation occur before the formation but after the destruction of myelin, which suggests that the maintenance of myelin is incompatible with the mobilized state of the Schwann cell. However, Nathaniel and Pease (1963) observed that myelin debris remains for up to 3 months after nerve injury.

Axonal sprouting.

Axonal outgrowths from the proximal stump can appear as early as 24 hours after nerve injury (Seckel, 1990). A number of branches project from each regenerating axon and traverse the nerve gap (Seddon, 1972). Haftek and Thomas (1968) found that after nerve crush, the basement membrane tubes remained intact. They also observed that Schwann cells and regenerating axons grew into the tubes. These findings led to the proposal that the tubes provide pathways that guide regenerating axons. Numerous sprouts can enter each basement membrane tube (Holmes & Young, 1942), some of which may coalesce. Some collateral sprouts degenerate whereas others undergo a rapid increase in diameter. Myelin sheaths are evident approximately 1 week after nerve transection. Axon innervation of the distal stump is random and removal of the distal organ

has been found to affect the maturation of fibres but not affect nerve sprouting.

Formation of perineurium.

Between 5 and 30 days after nerve transection, there is an increase in the number of cells in the endoneurial spaces which have been described as being flattened cells with irregular outlines and containing small globules (Allt, 1976). By 20 days, these cells migrate centripetally to lie beneath the perineurium forming an almost continuous subperineurial layer. After removing a small section of rat sural nerve, Thomas and Jones (1967) observed cells morphologically similar to fibroblasts surrounding groups of Schwann cells and their associated axons. These cells assumed the appearance of perineurial cells. Consequently, the regenerating axons in the region of the nerve gap became surrounded by perineurial cells such that mini-fascicles were formed. It is now accepted that the perineurium has a homeostatic function that contributes to the maintenance of constant composition of the endoneurial matrix.

Neurotrophic and neurotropic factors.

Different factors which influence the survival, sprouting and directional growth of regenerating nerve fibres have been recognized in recent years. One group of factors which promote the survival and growth of regenerating axons are the neurotrophic factors, of which, nerve growth factor (NGF) is the most well known. NGF and NGF receptors have been found to be synthesized by Schwann cells distal to the site of nerve injury and it has been proposed that external display of NGF on the membranes of Schwann cells can guide regenerating axons (Taniuchi, Clark, Schweitzer, Johnson, 1988). In addition, NGF has been found to be produced by tissues that are innervated by sensory and sympathetic neurons (Yankner &

Shooter, 1982). Other recognized neurotrophic factors include ciliary neurotrophic factor (CNTF) and motor nerve growth factor (MNGF).

Neurite-promoting factors, including laminin and fibronectin, are substrate-bound glycoproteins which promote initiation of neurite outgrowth and extension of the neurite outgrowths (Seckel, 1990). Laminin and fibronectin are components of endoneurial basement membranes and the experiments of Ide, Osawa and Tohyama (1990) demonstrated in experiments using freeze-killed nerve grafts in mouse sciatic nerve, that basement membrane tubes serve as effective conduits for regenerating axons. Basement membrane tubes of muscle cells have also been found to provide a suitable scaffold for regenerating axons (Ide, 1984). The freeze-thawed skeletal muscle autografts of Glasby (Glasby, 1990; Glasby, Gschmeissner, Hitchcock and Huang, 1986) probably provide a suitable conduit for regenerating nerve because of the presence of neurotropic factors in the basement membrane tubes of muscle grafts.

Other factors which have been found to promote nerve regeneration include gangliosides, thyroid hormone, adrenal hormone, sex hormones and insulin (Seckel, 1990). Kanje, Skottner, Sjöberg and Lundborg (1989) observed that insulin-like growth factor I stimulated the regeneration of rat sciatic nerve and Glazner, Lupien, Miller and Ishii (1993) found that insulin-like growth factor II increased the rate of regeneration of sensory axons in the sciatic nerve of rats and that endogenous insulin-like growth factors support the spontaneous regeneration rate of peripheral nerve.

Substances which exert an attractive influence on regenerating axons are called neurotropic factors. Specificity of regeneration requires the production of neurotropic factors which act at a distance to influence the direction of axonal growth. The types of specificity of nerve regeneration which have been proposed to exist are discussed in chapter 5.1.5. To what

degree specificity of growth occurs in nerve regeneration is highly debateable. It is known from the use of cutaneous nerve grafts in surgery of the peripheral nerve that motor axons can regenerate down endoneurial tubes of sensory nerves, however, Banks and Barker (1989) found that regenerating γ efferent axons were rarely seen entering muscle spindles via pathways initially occupied by sensory axons. Also, Brushart and Seiler (1987) demonstrated that regenerating motor neurons will preferentially reinnervate a distal motor branch.

1.2 Factors Affecting Nerve Regeneration.

The recovery of function after nerve injury and repair depends on the site and severity of the injury. There are two classifications of nerve injury which are currently used and describe the nature of the lesion. Seddon (1943) classified nerve injuries into 3 types: neurapraxia, axonotmesis and neurotmesis. The Sunderland classification categorizes nerve injuries into 5 types depending on the degree of nerve damage and is, perhaps, preferable because it provides greater information about the extent and implications of a nerve injury than the 3 types formulated by Seddon (Sunderland, 1990). Mackinnon and Dellon (1988) inserted a sixth 'injury' into Sunderland's original classification which is described below. Theoretically, the type VI injury does not fit Sunderland's classification because it describes a function of repair and not the injury itself.

Neurapraxia.

(a) Sunderland type I nerve injury.

When trauma to a peripheral nerve occurs such that there is local conduction block, which returns to normal over time (from minutes to weeks), the injury is termed neurapraxia or Sunderland type I nerve injury. In this type of injury there is no Wallerian degeneration.

Axonotmesis.

(a) Sunderland type II nerve injury.

In this type of nerve injury the damage is axonal in nature. The endoneurium and perineurium remain intact and full restoration of function usually occurs because the axon sprouts can regenerate down intact endoneurial basement membrane tubes and reach their original end organs (Sunderland, 1990).

(b) Sunderland type III nerve injury.

Unlike the Sunderland type II injury, there is loss of continuity of the endoneurial sheath. The injury to the nerve results in haemorrhage, oedema, inflammation and scarring in the endoneurium. Depending on the amount of scar tissue and the subsequent inhibition of regenerating axons, there is a wide range in recovery from very poor to very good (Mackinnon & Dellon, 1988).

(c) Sunderland type IV nerve injury.

In this type of injury the scarring in the endoneurium is so extensive that the scar tissue provides the only continuity between proximal and distal stumps. The problem for the surgeon is differentiating between Sunderland type II and type IV injuries because the former are best left to regenerate without surgical intervention, yet, after a type IV injury, the patient will only benefit if the nerve is transected, the scar tissue is removed and the nerve is repaired. Often the only way that a surgeon can diagnose the degree of injury is by leaving the nerve; after a Sunderland type IV injury there will be no recovery by 3 months.

Neurotmesis.

(a) Sunderland type V nerve injury.

This type of nerve injury is a simple transection injury where the nerve has been accidentally severed.

(b) Sunderland type VI nerve injury.

This type of 'injury' is sometimes termed neuroma-in-continuity which supposes that some regeneration has taken place at the time of diagnosing the injury otherwise there would be no neuroma present. Damage to the nerve is mixed and can vary from conduction block to transection within the same nerve. Obviously this type of nerve injury provides a great problem for surgeons in deciding how to operate.

Site of nerve injury.

The prognosis for a particular nerve injury after its repair depends on the site of the injury. The outcome after repair of proximal nerve injuries is often poorer than after similar repair of injuries occurring more distally. The reason for this is partly due to the distance over which the nerve fibres must regenerate to reach end organs. The degeneration of distal endoneurial tubes is more likely after a proximal injury, thus removing neurite-promoting factors and contact guidance for the regenerating axons. Nerve fibres are often arranged, in distal nerve, into motor and sensory fascicles, whereas the fascicles of more proximal nerve are often mixed sensory and motor (Sunderland, 1990). Therefore, the probability of 'crosswiring' is greater after repair of more proximal lesions. Also, the likelihood of death of nerve cell bodies is greater after more proximal nerve injury (Mackinnon & Dellon, 1988).

1.3 Types of Nerve Repair.

Nerve injuries of the Sunderland types IV to VI require surgical intervention. Historically, there have been some truly bizarre methods of nerve repair with surprisingly good results. Assaky (1886) tried joining proximal and distal stumps after nerve transection with suture scaffolds. Where proximal and distal stumps were separated by a gap, Assaky (1886) also tried stretching the nerve so that the stumps would meet. It is now accepted that regeneration of nerve is poor if a nerve has been stretched and sutured (Millesi & Meissl, 1981). This is probably because the *vasa nervorum* are flattened and the decreased blood supply to the nerve affects regeneration. In fact, Clark, Trumble, Swiontkowski and Tencer (1992) demonstrated that elongation of the sciatic nerve in rats by 8% and subsequent suture, caused a sufficient decrease in blood flow that was likely to cause ischaemia detrimental to nerve regeneration, and elongation of the nerve by 15% caused the sutures to be pulled out under the tension.

Nerve-to-nerve epineurial suture.

After clean nerve transection injuries (Sunderland type V) such as those caused by a razor or other sharp implement, it is often possible to suture the epineurium of the two stumps such that there is no tension on the nerve. Research has shown that recovery after nerve injury and repair is best if only one suture line is used in the repair, as compared to the two suture lines used in nerve grafts (Gutmann & Sanders, 1943), and this is true regardless of the length of the nerve graft (Banks, Barker and Brown, 1985). This may be because the amount of scarring is less when only one suture line is used in the repair and, therefore, there is less inhibition of the regenerating axons. Seddon (1972) argued against the proposal that invasion of the suture line by fibroblasts could lead to deposition of collagen

that was harmful to regeneration because of the rich supply of fibroblasts already present in nerve that proliferate and deposit collagen after nerve transection. But he stated that producing a suture line is, at the same time, also producing an injury and inevitably an inflammatory reaction will ensue. Two suture lines would necessarily produce greater oedema that could promote irregularity of axonal regeneration.

Improvements in the recovery of nerve after surgical repair has occurred in recent years with improvements in microsurgery. Surgeons now have better instrumentation, greater magnification and the availability of smaller sutures which induce less inflammation and subsequent scarring. The benefits of performing fascicular repair is still debated. It would seem sensible to join proximal and distal stumps of sensory or motor fascicles so that regenerating axons have a greater probability of reaching appropriate targets. The potential for 'cross-wiring' would seem to be greater after epineurial suture of the complete distal and proximal stumps. However, the disadvantages of fascicular repair include the inevitable increase in the inflammatory reaction which results from the use of additional sutures in the repair. The time taken in the operating theatre for fascicular repairs is longer and there is always a chance of fascicles being mismatched which might actually produce poorer results than epineurial suture of the complete nerve.

Nerve grafts.

When scar tissue, neuroma and/or glioma are removed, or if damage to the nerve is extensive and a section of nerve has to be removed, the gap between proximal and distal nerve stumps is generally bridged with a nerve graft.

Nerve grafts can be divided into three types:

(a) Xenografts.

This type of graft involves the use of pieces of nerve from individuals of different species.

(b) Allografts.

Nerve to make the graft is removed from individuals of the same species.

(c) Autografts.

This type of graft is made from nerve removed from the same individual.

Scepticism about the usefulness of nerve grafts remained until the 1970s, probably because the different types of nerve graft were considered together resulting in mixed reports of success. In surgical practice, nerve grafts are generally autografts formed from cutaneous nerves such as the sural nerve. In the repair of large mixed nerves it is often necessary to used a number of parallel lengths of smaller cutaneous nerve to make a cable graft of equal thickness to the nerve being repaired and, hence, provide sufficient numbers of endoneurial tubes for guidance of regenerating axons. Pover and Lisney (1989) found by suturing large and small size grafts into gaps in the sural nerve of cats that the number of available endoneurial basement membrane tubes did not affect the success of nerve regeneration. Myles and Glasby (1991) found that nerve regeneration and recovery of function was poorer after cable graft than either nerve-to-nerve suture or muscle graft in rats. They believe this was probably owing to infiltration of connective tissue into cable grafts and the subsequent impedance of pioneering axons. Glasby, Gilmour, Gschmeissner, Hems and Myles (1990) found that more nerve fibres could be counted distal to muscle grafts than cable grafts, after the repair of sheep femoral nerve.

The disadvantages in using nerve grafts include the inevitable loss of sensation from where the cutaneous nerve was removed to make the nerve graft. If nerve damage is such that a very long nerve graft is required, the removal of enough cutaneous nerve to make the graft may pose a problem. In the repair of cutaneous nerves, removal of a second cutaneous nerve to make a nerve graft to repair the first is rarely considered worthwhile.

Muscle grafts.

There is currently an increase in the use of prosthetic and bioprosthetic substances in nerve repair (Glasby, 1990). Kilvington used fresh muscle in experiments to bridge nerve gaps as early as 1908, before nerve grafts were accepted as being useful in the repair of peripheral nerve, but had no success (reviewed by Glasby & Hems, 1993). The use of freeze-thawed skeletal muscle autografts in the repair of peripheral nerve was pioneered by Glasby (Glasby, 1990). Osmotic disruption of the tissue components of skeletal muscle is produced by freezing the muscle in liquid nitrogen and thawing it in distilled water; this process leaves the basement membrane tubes intact which then act as a scaffold for axonal regeneration. Glasby, Gschmeissner, Hitchcock, Huang and de Souza (1986) demonstrated that nerve regeneration through freeze-thawed skeletal muscle autografts in rats, as assessed morphologically and electrophysiologically, was not significantly different from regeneration through nerve grafts. Functional recovery has also been shown to be good after using muscle grafts in the repair of peripheral nerve: by the demonstration of the recovery of functional motor endplates (Gattuso, Davies, Glasby, Gschmeissner and Huang, 1988; Gschmeissner, Gattuso, Glasby and Huang, 1988) and the return of response of low-threshold cutaneous mechanoreceptors to stimulation (Findlater, Reichert and Glasby,

1990). Freeze-thawed skeletal muscle autografts have been used in the repair of peripheral nerve in rats (Myles & Glasby, 1991), sheep (Glasby *et al.*, 1990; Glasby, Mountain and Murray, 1993) and primates (Gattuso *et al*, 1988; Glasby, Carrick and Hems, 1992). The successful regeneration of peripheral nerve through muscle grafts led to the proposal that there may be a place for muscle grafts in the repair of human nerves. Norris, Glasby, Gattuso and Bowden (1988) demonstrated the effectiveness of freeze-thawed skeletal muscle autografts in the repair of digital nerves in humans.

The advantages of using muscle grafts include the minimalization of iatrogenic morbidity. The amount of muscle removed to make a muscle graft is unlikely to affect the performance of the muscle as a whole; this is in contrast to the loss of sensation which occurs as a result of removal of the cutaneous nerve necessary to make a nerve graft. Simpson and Young (1945) and Berry and Hinsey (1946) demonstrated that narrow diameter tubes of Schwann cell basement membrane have a restricting effect on the development of regenerating axons of large diameter. Although this view is somewhat debateable, muscle-derived basement membrane tubes are large enough to accomodate the largest fibres (Glasby, 1990). In contrast, the diameter of fibres of cutaneous nerves are narrow in comparison to the diameters of fibres of large mixed nerves, and consequently, the endoneurial basement membrane tubes of nerve grafts, made from cutaneous nerve, could have a restricting effect on the regeneration of large diameter axons through the graft.

Poorer recovery after regeneration through long muscle grafts (5 cm) has been reported in rabbits by Hems and Glasby (1992). The reason for the poorer regeneration through long muscle grafts in contrast to the quite successful regeneration of nerve through short nerve grafts, may well be

due to the lack of Schwann cells whose trophic influences will inevitably be lacking in the former. This suggestion seems likely considering the work of Nadim, Anderson and Turmaine (1991) who found that regeneration through 4 cm freeze-killed and, therefore, acellular nerve grafts, in rats, were not successful. Although the potential for the use of freeze-thawed skeletal muscle autografts in large nerve defects is not great, there certainly seems to be a place for muscle grafts in the treatment of painful neuromata and digital nerve injuries.

1.4 Diabetes Mellitus and Nerve Regeneration.

In addition to exploring recovery of peripheral nerve after the various methods of injury and repair described above, recovery was compared to animals with a superimposed metabolic disorder. A hyperglycaemic state similar to that seen in diabetes mellitus was produced in half of the rats used in the experiments presented in this thesis by administration of the compound streptozotocin (see chapter 2.2). The reasons for creating this particular metabolic state in the animals of the work presented here are discussed below.

Many metabolic abnormalities cause neuropathy including excess alcohol and vitamin deficiency (thiamine or vitamin B1 and Pyridoxine or vitamin B6); hereditary disorders of lipid metabolism (e.g. Tangier disease, Bassen-Kornzweig disease, sulphatide lipidosis) and porphyrias. The most commonly encountered metabolic neuropathy is diabetic neuropathy.

Evidence of neuropathy is found in both type I or insulin-dependent (IDDM) and type II or non-insulin dependent (NIDDM) diabetes mellitus; the reported incidence of neuropathy in diabetic patients varies from 12 to 50% (Masson & Boulton, 1990). It is estimated that one half of diabetics receiving insulin treatment have a symptomatic neuropathy (Sumner, 1991), however, patients with nerve abnormalities may not have any symptoms (Masson & Boulton, 1990). The lack of symptoms may obscure the true incidence of neuropathy in the diabetic population; Fraser, Cambell, Ewing, Murray, Neilson and Clarke (1977) claimed that electrophysiological evidence of abnormal peripheral nerve function can be obtained in nearly all diabetic subjects. In diabetic neuropathy all fibre types may be affected and show axonopathic degenerative changes; demyelination and remyelination are also encountered in myelinated fibres (Masson & Boulton, 1990). In the

treatment of nerve in leprotic patients where nerve is already damaged as a result of the pathological processses of the disease, Pereira, Palande, Subramanian, Narayanakumar, Curtis and Turk (1991) found that muscle grafts used to repair the tibial and median nerves of 10 patients with leprosy resulted in the recovery of sensation in 7 cases. The sacrifice of cutaneous nerves to form nerve grafts, in a situation where loss of function of major nerves has already occurred, may not be justifiable. As a result of the pathological processes of the disease, the cutaneous nerves themselves may be inappropriate for use as nerve grafts.The problems associated with repairing peripheral nerves in diabetic patients with nerve grafts made from damaged cutaneous nerve has not been investigated.

Given that the estimated incidence of symptomatic diabetes mellitus in the adult population of the United States is 1 to 2% (Robbins & Kumar, 1987) and that the incidence of IDDM appears to be increasing in Northern Europe and NIDDM is increasing in all populations of the developed nations (Gale & Monson, 1990), the probability of a diabetic suffering a nerve injury is sufficiently high to make it worthwhile investigating nerve injury and repair in this group of patients.

An advantage of investigating nerve repair in animals with diabetes mellitus is the ease with which the diabetic state can be induced. In the work presented here, the broad spectrum antibiotic streptozotocin was used to induce destruction of the β cells of the islets of Langerhans. This compound was first reported by Rakieten, Rakieten and Nadkarni (1963) to be diabetogenic in dogs and rats; since then many researchers have used the drug to produce a diabetic state in experimental animals which is similar to IDDM or type I diabetes mellitus.

The difficulty with any experimental animal model is in determining how accurately the animal situation reflects the human one and thus drawing

well-founded and informative parallels from the experimental results. In the case of peripheral nerve regeneration in the rat, the problems are mainly due to the rapidity with which rat nerve regenerates and, because of the size of the animal, the short distance over which regeneration takes place. The figure often quoted for rate of regeneration of the human peripheral nerve is 1 to 1.5 mm day^{-1} (Seddon, Medawar and Smiths,1943), whereas the rate of axonal elongation of the rat sciatic after crush injury is approximately 3.6 mm day^{-1} (Black & Lasek, 1979). It is known that the success of regeneration in the human depends on the site of the lesion; a more distal lesion has a better prognosis of recovery (Mackinnon & Dellon, 1988). The nerve injuries inflicted on the rats used in the work presented here were never more than 10 cm from the end organ. Therefore, 150 days after operating (i.e. the time allowed to ellapse, in the experiments of this thesis, for regeneration to occur), recovery is likely to be almost complete and differences in recovery between experimental groups may not be clear. Nerve regeneration in diabetic animals has been demonstrated to be slower than in non-diabetic animals (Longo, Powell, LeBeau, Gerrero, Heckman and Myers, 1986; Ekström & Tomlinson, 1989) and it was thought that such a superimposing metabolic disorder might delay nerve regeneration in the rat, thereby effectively adding 'distance' over which the nerve had to regenerate. In this way any differences in recovery after different types of nerve injury and repair would more likely be exposed.

1.5 Diabetes.

Diabetes is divided into two types: insipidus and mellitus. In the diabetic state there is passage of excess urine; in diabetes mellitus this is associated with hyperglycaemia. The passage of excess urine in diabetes insipidus is either due to lack of anti-diuretic hormone (ADH) or ADH

resistance of the nephrons. Diabetes mellitus can be further divided into primary and secondary types. The secondary type of diabetes mellitus arises in association with other diseases e.g. Addison's disease, Cushing's disease, phaeochromocytoma. The work presented here is only relevant to primary diabetes mellitus which itself can be divided into two types: type I, juvenile onset or insulin-dependent (IDDM) and type II, maturity onset or non-insulin dependent (NIDDM).

Type I tends to be used synonymously with IDDM although many sufferers with type II diabetes mellitus need and use insulin treatment. Patients with IDDM form 15 to 20% of the population with diabetes mellitus. As the old classification suggests, this type of the disease commonly arises before or around the age of puberty. Insulin levels are characteristically low; this is now thought to be the result of autoimmune processes. Auto-antibodies targeted at the β cells of the islets of Langerhans in the pancreas are found in 70% of newly diagnosed young patients (Gale & Monson, 1990).

Type II diabetes mellitus affects 2% of the population of the United Kingdom (Gale & Monson, 1990). These patients generally present much later on in life than those with type I and approximately 60% of patients with type II are obese (Robbins & Kumar, 1987). Insulin levels are normal to high in type II diabetes mellitus and the inappropriately high levels of glucose in the blood are thought to be due to insulin resistance of peripheral tissues and abnormal secretion of insulin.

Diabetes was induced in the rats used in the experiments presented in this thesis by an intraperitoneal injection of streptozotocin (STZ). The type of diabetes induced in the rats used in the experiments presented here more closely resembles type I diabetes mellitus because the action of STZ causes destruction of β cells and thereby produces correspondingly low

levels of insulin production (see chapter 2.2). However, the aims of the experiments presented here were to investigate the effects of hyperglycaemia on nerve regeneration and not to investigate the causation of the hyperglycaemia. In this respect, the metabolic disorder induced in the animals used in the experiments presented in this thesis was comparable to both type I and type II diabetes mellitus, as both types are characterized by high levels of blood glucose.

1.6 Diabetic Neuropathy.

Neuropathy is a common complication of diabetes (see above). Many theories exist as to why neuropathy occurs, although the precise aetiology is still not fully understood. Part of the confusion lies in the fact that presenting neuropathies form a very heterogeneous group. Different systems for classifying diabetic neuropathy are commonly encountered in different textbooks. The classification given below has been produced by combining the systems used by Sumner (1991) and Gale (1990):

1. Distal symmetrical polyneuropathy
   (a) sensory; large fibre, small fibre or mixed type
   (b) mixed sensorimotor-autonomic
2. Focal and multifocal neuropathies
   (a) cranial
   (b) trunk and limb
   (c) proximal motor diabetic amyotrophy
3. Autonomic neuropathy
   (a) cardiovascular
   (b) gastro-intestinal tract
   (c) bladder
   (d) impotence

By far the commonest type of neuropathy in diabetes mellitus is the symmetrical sensory polyneuropathy (Gale & Monson, 1990). The incidence of neuropathy increases with duration of diabetes (Sumner, 1991), age and possibly poor glycaemic control (Masson & Boulton, 1990).

Neuropathic changes in diabetic nerve.

Peripheral nerve in experimental diabetes does not exhibit all of the changes that are observed in human diabetic neuropathy. For this reason, research into the pathogenesis of neuropathy and extrapolation of results from experimental diabetes to the human situation is not straightforward.

Two changes in nerve function that are seen in both humans and animals in the diabetic state are: a reduction in nerve conduction velocity and resistance to ischaemic conduction block (RICB).

Ward, Barnes, Fisher and Jessop (1971) demonstrated a return of conduction velocity to normal levels in newly diagnosed diabetics following establishment of glycaemic control. Eliasson (1964) showed that insulin treatment of alloxan diabetic rats had no effect on the reduced conduction velocities, although Jakobsen's (1979) STZ diabetic rats did show an improvement in conduction velocity following insulin treatment. Perhaps the difference in results could be due to the fact that the blood glucose of the animals used by Eliasson (1964) was not adequately controlled because he did not actually monitor the blood glucose levels and adjust the amounts of insulin given to each animal. Instead, an equal amount of insulin was given daily to each rat, regardless of the severity of hyperglycaemia.

RICB is seen in newly diagnosed patients but is reversed by euglycaemia (Thomas, 1990). RICB in animal models is discussed below but may be due to the increase in glycogen storage which occurs in diabetic

animals (Tuck, Schmelzer and Low, 1984). The glycogen could be used for the production of energy compounds, in situations of hypoxia, through its hydrolysis and subsequent passage of glucose into the glycolytic pathway.

Although similar functional changes are exhibited by human and experimentally diabetic nerve, many of the morphological changes observed in human diabetic nerve have not been confirmed in animal experimental models.

The earliest structural change that has been observed in rats, after the induction of the diabetic state, is nodal and paranodal swelling of the axon. These changes were seen in the spontaneously diabetic BB Wistar rat and have also been reported in human diabetic neuropathy (Sima, Yagahashi and Greene, 1990).

It is now generally accepted that axonal dwindling and segmental demyelination are neuropathic changes which occur in human diabetes. Changes in axonal and fibre diameters, indicative of the processes stated above, have been reported in both experimental and human diabetes. Jakobsen (1979) reported a decrease in cross-sectional area of myelinated fibres but no decrease in numbers of myelinated fibres in experimentally diabetic rats as compared with their non-diabetic controls. He also observed a decrease in the axon/myelin relationship in diabetic nerve suggesting that the decrease in cross-sectional area was due to a diminution in the size of the axonal area. Although Wattig, Warzok, Zglinicki and Hufnagl (1991) confirmed the findings of a decrease in cross-sectional area of myelinated fibres without a decrease in number of myelinated fibres, these authors failed to find a change in mean axonal area but rather, they found a reduction of myelin cross-sectional area. Sharma, Thomas and De Molina (1977) did not find any reduction in fibre diameter nor any alteration in the relationship between myelin sheath thickness and axonal circumference.

There was no loss of myelinated fibres of the sural, peroneal or tibial nerves in STZ diabetic or alloxanized rats according to Sharma & Britland (1990), although the "subtle morphological changes" such as those described above have been recognized in these particular animal models of diabetes.

Although the evidence of axonal dwindling and segmental demyelination remains somewhat debateable in animal models of diabetes, there is almost unquestionable evidence of these pathological processes occurring in humans (Greenbaum, Richardson, Salmon and Urich, 1964; Thomas & Lascelles, 1966). There is, however, some debate over which fibres are most commonly affected. Archer, Watkins, Thomas, Sharma and Payan's (1983) observations on sural nerve biopsies obtained from patients with acute painful neuropathy showed "evidence of active degeneration of myelinated nerve fibres of all diameters". Brown, Martin and Asbury (1976) showed a predominant loss of small diameter myelinated fibres in his nerve biopsies from patients with painful diabetic neuropathy.

Microangiopathy is seen in both diabetic humans (Yasuda & Dyck, 1987) and in experimental diabetes (Thomas, 1990), however, such changes are seen to a greater extent in the clinical situation (Low, Schmelzer, Ward and Yao, 1986). Thickening of the walls of endoneurial capillaries was first observed by Fagerberg (1956). This thickening has subsequently been shown to be due to a reduplication of the basal lamina of the vessels. Yasuda & Dyck (1987) demonstrated an increase in the number of endothelial nuclei, area of endothelium and thickness of basement membranes in the sural nerves of patients with diabetic neuropathy. They also showed that the incidence of capillary closure was greater in diabetics than their controls. In STZ diabetes rats, Maxfield, Cameron, Cotter and Dines (1993) found that the capillary density was not different to non-diabetic controls.

The presence of morphological changes occurring in animal models of diabetes may not, as yet, be confirmed but findings relating to changes in cell biochemistry and metabolism are more consistent. For ethical reasons, it is not always possible to perform the same tests on humans as on animals and the interpretation of data from animal experiments and their application to the human situation is only theoretical.

Schmidt, Matchinsky, Godfrey, Williams and McDougal (1975) demonstrated a decrease in both fast and slow components of axoplasmic flow by demonstrating the accumulation of acetylcholinesterase (AChE) and choline acetylase (ChAc), respectively, proximal to a tie placed around the sciatic nerve of STZ diabetic rats. Medori, Autilio-Gambetti, Monaco and Gambetti (1985) demonstrated a decrease in both components of slow axonal transport in STZ diabetic rats; SCa i.e. tubulin and neurofilaments, travelling at a rate of 1 mm day^{-1} and SCb i.e. actin and other soluble proteins, travelling at a rate of 3-4 mm day^{-1}. SCb was more severely affected. This is to some extent in agreement with the findings of Mayer, Tomlinson and Mclean (1984), who found a small but significant decrease in the rate of axonal transport of the tail of the slow component profile i.e. rates of 1-1.3 mm day^{-1} and Sidenius and Jakobsen (1982) who found that the decrease in the rate transport of SCa was preventable by insulin treatment. Medori *et al.* (1985) and Jakobsen and Sidenius (1980) have suggested that the decrease in axonal transport, particularly the transport of neurofilaments, could affect the calibre of axons. Thomas (1986) said that a symmetrical distal neuropathy is implicit of a "selective involvement of longer axons". It may be that a decrease in orthograde axonal transport could be responsible for the preferential vulnerability of distal axons.

One explanation for the vulnerability of longer axons in human diabetes is that an impairment in perikaryal synthesis results in a decrease

in axonal transport which leads to the inability of the cell to maintain axons in the periphery. Alternatively, longer axons may be more susceptible to multifocal lesions than shorter axons which results in greater cell death of the damaged long axons. Whatever the cause, the kind of lengths of axons seen in human nerve are not found in the animals which are most commonly used in investigations into experimental diabetes (i.e.rats, rabbits, mice) and it is, therefore, not surprising that similar pathological changes are not observed in these animal models. In contrast, similar metabolic changes are found in animal models of diabetes and it may be that some of the neuropathology that occurs in humans is not observed in animals simply because of anatomical differences.

The other major difference between experimental models and human diabetes is the duration of the metabolic disturbance in experiments. Although nerve conduction changes and RICB are observed early on in animal experiments, Jakobsen (1979) noticed a 3-4% reduction in conduction velocity as early as 24-36 hours after STZ administration; such changes in human nerve are reversible if the diabetes is diagnosed early and adequate glycaemic control is obtained (Ward, Bowes, Fisher, Jessop and Boher, 1971). Long-term disturbances in conduction velocity do not respond so rapidly to insulin treatment and may be due to degenerative changes in axons and demyelination which are linked to the duration of diabetes. Those pathological changes seen in the clinical situation which are not reproduced in animal models could be lacking in the latter because of the limited duration of animal experiments.

1.7 Pathogenesis of Diabetic Neuropathy.

The pathogenesis of diabetic neuropathy is not fully understood. Owing to the variety of neuropathic types, neurological changes probably

result as a combination of factors. It may be that the dominant factors which determine the development of neuropathology may be different for NIDDM and for IDDM. The predominant factor in the causation of neuropathy may also depend on the duration of diabetes. It is quite believable that vascular factors could be more important in neuropathy of patients with long-term NIDDM, for example, because of the probability of such patients having other vascular complications. The reversibility of the decrease in conduction velocity in short term or acute diabetes (as described above) may be due to different factors being operational at different stages of the disease.

The two most important of the hypotheses to explain the causation of diabetic neuropathy are the vascular and metabolic mechanisms.

Vascular hypothesis.

The idea that structural changes in the vasculature leads to hypoxia and neuropathology dates back to the late nineteenth century (Pryce, 1893). Woltman and Wilder (1929) also had ideas of large-vessel occlusive disease being the principal cause of diabetic neuritis. The work of the latter authors has been generally dismissed owing to the fact that their work was performed on tissue from autopsies or amputated limbs, in which atherosclerotic changes would not be unlikely (Thomas, 1990).

The vascular hypothesis is discussed in chapter 8 but, in brief, the hypothesis supposes that changes in peripheral nerve vasculature are primary to the development of neuropathy. It has been proposed that chronic hyperglycaemia leads to the development of microvascular changes via the production of advanced glycosylation products, and the consequent decrease in endoneurial blood flow and oxygen tension cause a chronic energy deficiency in the cells, resulting in the functional and

structural abnormalities characteristic of diabetic neuropathy (Tuck, Schmelzer and Low, 1984).

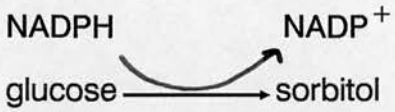
Winegrad & Simmons (1990) argued that decreased tissue oxygenation is unlikely to be the cause of diabetic neuropathy. They stated that should cells be chronically starved of energy, one would expect to see acute necrosis, yet this is not seen in diabetic neuropathy. It has been suggested that cells may be able to survive short-term energy deficiency by use of phosphocreatine stores for ATP production and a decrease in the biochemical activities of the cell. In addition, the shortage of endoneurial oxygen for oxidative phosphorylation could be counteracted by an increase in anaerobic production of energy compounds via the glycolytic pathway. However, Winegrad and Simmons (1990) argued that cells could not possibly survive long-term ischaemia because the lack of oxygen would result in tissue acidosis due to the inability of the cells to oxidize the lactic acid produced through anaerobic respiration. The authors claimed that if the vascular hypothesis were the major cause of neuropathy, one would expect to see changes in the phosphocreatine/creatine ratio (as this is a sensitive indicator of energy balance in nerve) and these have not been observed.

Evidence in favour of the vascular hypothesis comes from the existence of focal and multifocal neuropathies. Localized occlusion of vessels could lead to degenerative changes in specific nerves. Some patients experience an acute onset of nerve problems which subsequently recover, for example, third cranial nerve lesions, which would be consistent with a vascular aetiology of neuropathy. In contrast, Thomas (1990) stated that other ischaemic neuropathies in man do not show a predominantly sensory and autonomic disturbance as is found diabetic neuropathy.

Metabolic hypothesis.

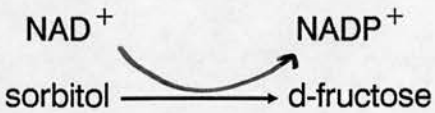
Those researchers who regard the metabolic causation of diabetic neuropathy to be the most significant, believe that changes in the metabolic activities of nerve, as a result of hyperglycaemia, are the primary factors in the causation of neuropathy. The metabolic hypothesis is outlined below.

Importantly, peripheral nerve is insulin-independent for the uptake of glucose. For this reason, nerve glucose concentration increases in a parallel way to blood glucose concentration (Gabbay, 1973). Aldose reductase is the rate-limiting enzyme in polyol metabolism. This enzyme has low affinity for glucose but in hyperglycaemia, the sugar is reduced to sorbitol.



aldose reductase

Sorbitol is further reduced to fructose.



sorbitol dehydrogenase

There is, therefore, an increase in the cellular metabolites, sorbitol and fructose, in the hyperglycaemic state. The increase in polyol metabolism has been linked to a depletion in nerve myoinositol, possibly by inhibition of active myoinositol transport by glucose in the endoneurium (Greene & Lattimer, 1982). Greene, Lattimer and Sima (1988) proposed ways in which myoinositol depletion and disturbances in phosphoinositide metabolism could cause structural damage of peripheral nerves. They stated that disruption of Na^+/K^+ ATP-ase function has been implicated in the causation of a four-fold rise in endoneurial Na^+ concentration and

subsequent decrease in nerve conduction velocity. The findings of Greene, DeJesus and Winegrad (1975) showed that insulin treatment or supplementation of dietary myoinsitol produced normalization of myoinositol levels and a recovery of decreased conduction velocity in STZ diabetic rats. Therefore, if myoinositol depletion is involved in the decrease in conduction velocity, it must somehow cause disruption of Na^+/K^+ ATP-ase function. Greene *et al.* (1988) found from previous studies that normalization of Na^+/K^+ ATP-ase activity was produced by normalization of myoinositol levels either with myoinositol supplementation or with aldose reductase inhibitors. The aforementioned authors proposed a possible mechanism for the action of myoinositol in causing the disruption of Na^+/K^+ ATP-ase. Myoinositol is incorporated into phosphoinositides whose turnover regulates intracellular mediators: diacylglycerol and water-soluble inositol phosphates. The latter compounds, in turn, affect protein kinase C activity and intracellular Ca^{2+} translocation. A decrease in protein kinase C activity is very likely to cause a reduction in Na^+/K^+ ATP-ase activity.

Similar decreases in nerve myoinositol have not been shown in humans. In fact, Dyck, Zimmerman, Vilen, Minnerath, Karnes, Yao and Poduslo (1988) failed to find such a depletion of endoneurial myoinositol in diabetic patients, with and without neuropathy. Mayhew, Grillon and Hawthorne (1983), however, did demonstrate a reduction of myoinositol concentations in human diabetic nerve. The arguments in favour of the metabolic hypothesis are the same as those against the vascular hypothesis and include the fact that there is selective affection of sensory and autonomic fibres. Also, there is no direct proof that the microvascular changes which are known to occur in diabetes, are primary to neuropathy.

Tomlinson, Moriarty and Mayer (1984) found that increase in polyol metabolism is associated with reduced Na^+/K^+ ATP-ase activity and reduced axonal transport of proteins in STZ diabetic rats. The aforementioned authors observed that both the reduced Na^+/K^+ ATP-ase activity and decrease in protein axonal transport were prevented with the aldose reductase inhibitor, sorbinil. Such findings could implicate the role of a disturbance in polyol metabolism and subsequent disruption of cellular myoinositol and phosphoinositde metabolism in nerve regeneration. However, not all components of axonal transport are improved by treatment with aldose reductase inhibitors (Tomlinson, Sidenius and Larsen,1986).

1.8 Nerve Regeneration in Diabetes.

There are conflicting reports of the effect of diabetes on the regeneration of peripheral nerve. Both Sharma and Thomas (1975) and Eliasson (1965) failed to find any reduction in regenerative capacity of peripheral nerve in animal models of diabetes. However, other groups of researchers have shown a reduction in the capacity of peripheral nerve to regenerate in diabetes (Longo *et al.*, 1986; Ekström, Kanje and Skottner, 1989; Ekström & Tomlinson, 1989).

Sharma and Thomas (1975) measured the average fibre diameter of the sural nerve in STZ diabetic rats 3, 6 and 18 weeks after a nerve crush injury. They showed no difference in the average fibre diameter between diabetic and control animals; however, they did show a change in the linear relationship between myelin sheath thickness and axon circumference at 3 weeks after nerve crush. The change was indicative of a delay in maturation of peripheral nerve in diabetic animals but this difference was not apparent in the rats left for longer periods of time after nerve crush. Triban, Guidolin, Fabris, Marini, Schiavinato, Dona, Bortolami, Di Giamberardino and Fiori

(1989) showed a reduced number of regenerating axons in alloxan diabetic rats after crushing the sciatic nerve and demonstrated this to be almost completely prevented when treatment with gangliosides was given.

Although Sharma and Thomas (1975) did not demonstrate a difference in nerve regeneration in STZ diabetic rats, Ekström and Tomlinson (1989) showed a decrease in the rate of advancing axon tips in STZ diabetic rats 7 days after a nerve crush injury. The latter authors also found that the use of an aldose reductase inhibitor had no effect in preventing the decreased rate of regeneration, suggesting that the reduced capacity of diabetic nerve to regenerate was not due to an increase in the sorbitol pathway flux. Ekström *et al.* (1989) have also shown a decrease in the rate of regeneration of peripheral nerve in STZ diabetic rats after nerve crush injury with a concomittant decrease in serum levels of insulin-like growth factor-1 (IGF-1). These authors demonstrated that insulin treatment of their STZ diabetic rats corrected both the reduced rate of regeneration of the sciatic nerve and also restored levels of IGF-1 to normal levels.

In contrast with the findings of Ekström *et al.* (1989) and Ekström and Tomlinson (1989), Bisby (1980) did not show a decrease in the rate of nerve regeneration in his STZ diabetic rats but he did demonstrate a decrease in the initial delay before nerve regeneration attained a constant velocity. Longo *et al.* (1986) observed the regeneration of axons (and the extent of myelination) of fibres regenerating through 1 cm long silicone tubes which were placed between the stumps of sectioned sciatic nerves in STZ diabetic rats. They found that the axons in non-diabetic animals bridged the 1 cm gap within 3 weeks, with myelination of the fibre extending for 0.6-0.8 cm. After the same amount of time, the axons and myelin sheaths in the diabetic animals had regenerated no more than 0.2 cm into the silicone tubes. The

axons in the diabetic animals crossed the 1 cm gap by 4 weeks but appeared "immature and showed dystrophic changes".

Although the literature on nerve regeneration in diabetes is somewhat scarce, the current view seems to be an acceptance that peripheral nerve in STZ diabetic rats shows a decreased capacity to regenerate and that this decreased rate is not due to the increased sorbitol pathway flux (Ekström & Tomlinson, 1989). The possible mechanisms behind the decreased rate of peripheral nerve regeneration observed in diabetics are discussed below.

The rate of axonal regeneration has been correlated with SCb of axonal transport (Wujek & Lasek, 1983). Certain defects in axonal transport have been reported in experimental diabetes. Schmidt, Matschinsky, Godfrey, Williams and McDougal (1975) demonstrated reductions in both fast and slow axoplasmic flow in STZ diabetic rats by measuring the activity of acetylcholinesterase and choline acetylase proximal to a tie placed around the sciatic nerve in STZ diabetic rats. Mayer, Tomlinson and McLean (1984) showed a decrease in the rate of transport of only the most slowly transported proteins in the motoneurones of the sciatic nerve. Both Jakobsen and Sidenius (1980) and Sidenius and Jakobsen (1982) found a decrease in slowly transported proteins in sensory fibres of STZ diabetic rats. In contrast, Medori *et al.*, (1985) demonstrated a decrease in the transport of constituents of both SCa and SCb of axonal transport. They showed that the transport of constituents of the SCa (including neurofilament subunits and tubulin) to be affected to a greater degree than the transport of the constituents of SCb (including some 30 and 60 kDa polypeptides and actin). In addition, they suggested that the decrease in slow axonal transport correlated with a decrease in axon diameter, something which was also postulated by Jakobsen and Sidenius (1980).

Gangliosides are naturally occurring glycosphingolipids which are particularly abundant in the membranes of neural cells (Triban *et al.*, 1989) and have been shown to increase the number of regenerating fibres after a crush injury of the sciatic nerve in rats (Sparrow & Grafstein, 1982). Calcutt, Tomlinson and Willars (1988) demonstrated that the treatment of diabetic rats with gangliosides prevented the deficit in the slow transport of 6-phosphofructokinase, while Marini, Vitadello, Bianchi, Triban and Gorio (1986) demonstrated that the treatment of diabetic rats with gangliosides protected against the decreased slow axonal transport of acetylcholinesterase.

Ekström and Tomlinson (1989;1990) demonstrated that ganglioside treatment of STZ diabetic rats, started 7 days prior to crushing the sciatic nerve, attenuates the reduction in the rate of advancement of axon tips. Triban *et al.* (1989) also showed the positive effects of ganglioside treatment; the reduced number of axons able to regenerate in their alloxan diabetic rats was almost completely compensated for by the treatment with gangliosides.

Sidenius and Jakobsen (1982) suggested that the decrease in the SCa component of axonal transport seen in diabetic rats could be accounted for by a smaller production of protein in the nerve cell body. The reduction in the delivery of protein to the axon intended for transportation to the periphery, may be due to a decrease in the enzymic activity within nerve cell bodies of diabetic animals. Having found that the increase in the activity of the enzyme ornithine decarboxylase (ODC) in the dorsal root ganglia after nerve crush injury of the sciatic nerve was much higher in non-diabetic than diabetic rats, McLean, Chapman and Cullum (1987) postulated that this could be responsible for delayed and defective regeneration of peripheral nerve in diabetic rats.

Ornithine decarboxylase is the initial and rate-limiting enzyme in the polyamine biosynthetic pathway. Production of the diamine putrescine from ornithine is catalysed by ODC. Putrescine is a growth factor and accumulates in tissues in response to a growth stimulus and is the precursor of spermine and spermidine. Spermidine increases the rate of chain elongation of RNA and DNA and it also increases the rate of protein synthesis. Spermine increases the efficiency of acylation of transfer RNA (tRNA) which is required for optimal protein synthesis (Russell, 1985). Evidence exists for the post-translational modification of ODC after translation by the incorporation of putrescine into its structure (putrescine is attached to the glutamine residues of ODC). In so doing, ODC loses its enzymatic activity and the new complex stimulates nuclear RNA polymerase I activity. RNA polymerase I is rate-limiting for the synthesis of ribosomal RNA (rRNA). The effects of a decrease in the activity of ODC, such as are seen in the dorsal root ganglia of STZ diabetic rats after nerve injury, would be expected to be reflected in nucleic acid and protein synthesis and hence, the manufacture of substances essential to nerve regeneration.

The mechanism behind the stimulation of ODC is as yet unknown, however, it has been postulated that a retrogradely transported signal from the site of injury to the cell body stimulates the enzyme and that this signal could be NGF, insulin or possibly IGF-1 (Kanje, Fransson, Edström, Löwkvist, 1986). Poor retrograde transport of NGF and insulin deficiency are features of STZ diabetes (Jakobsen, Brimijoin, Skau, Sidenius and Wells, 1981). Growth hormone, administered to adult and weanling rats, is known to increase the activity of ODC by a cAMP-dependent mechanism; NGF and insulin are also known to increase ODC activity by an unknown mechanism (Russell, 1985). Sköttner, Kanje, Widegran, Lundborg, and Lofberg (1987)

have implicated the role of IGF-1 in peripheral nerve regeneration as have Hansson, Dahlin, Danielson, Frykland, Nachemson, Polleryd, Rozell, Sköttner, Stemme and Lundborg (1986) and the latter group of researchers have presented evidence that, in the sciatic nerve of rats, IGF-1 is mainly formed by Schwann cells.

In summary, the possible reason for a reduced capacity of peripheral nerve in diabetics to regenerate, is that poor signalling to the cell bodies of regenerating nerve fibres results in diminished activity of certain enzyme(s) involved in nuclear metabolism; this, in turn, results in insufficient production of some of the protein components required for regeneration and which is transported at a decreased axonal rate, as compared to non-diabetics.

CHAPTER 2

General Materials and Methods

2. General Materials and Methods.

2.1 Preparation of Experimental Animals.

Male Sprague-Dawley Rats were bought at weights of approximately 350 gwt and housed in the Medical Faculty Animal Area. Rats at this weight are 10-12 weeks old and are considered by the supplier to be adult (Bantin and Kingman, Universal Ltd, Hull, U.K.). In their work on rats of different ages, Bharali and Lisney (1990) considered that their 13 week old rats were post-pubertal. The animals were allowed a period of one week to adjust to the new environment as recommended in the University Federation for Animal Welfare (U.F.A.W.) Handbook (1989). They were given the 'Modified Maintenance Diet' (Special Dietary Supplements (SDS), Essex, U.K.), *ad libitum*. This is a low protein diet designed for use in prolonged experiments to reduce obesity and decrease the probability of renal dysfunction which is often encountered in long term trials (SDS). The components of the diet are listed in appendix 1.

The animals were kept three per cage as recommended in the U.F.A.W. Handbook, 1989. Each cage was supplied with one bottle of water, except in the case of the diabetic animals who had two bottles per cage owing to the fact that these animals showed a significant increase in thirst (see below). There was also a noticeable increase in appetite in the diabetic animals making it necessary to replenish their food at more frequent intervals (see below). Both food and water were checked each morning by the Medical Faculty Animal Area staff. Polyuria made it necessary to change the bedding and cages of the diabetic animals each day.

2.2 Induction of Diabetes Mellitus.

In each experiment, half of the animals had diabetes induced with a single dose of 55 mg kg^{-1} of streptozotocin (Sigma Chemical Company Ltd, Poole, Dorset, U.K.) in citrate buffer which was injected intraperitoneally (see below).

Streptozotocin (STZ) is a broad spectrum antibiotic and a by-product of the bacterium Streptomyces achromogenes; reported by Rakieten *et al.* (1963) to be diabetogenic in dogs and rats. As a result of STZ administration, few β cells survive. The relative insulin deficiency leads to permanent diabetes and hyperglycaemia (Brosky & Logothetopoulos, 1969). The resulting type of diabetes is closest to type I or juvenile diabetes mellitus as insulin levels are abnormally low; in type II or late-onset diabetes this is not necessarily the case (see chapter 3).

Patients with diabetes mellitus often present with polydipsia and polyuria; similarly animals show both these signs 12 to 24 hours after STZ injection. These two signs arise as a result of the inability of the kidneys to reabsorb all the glucose from the fluid passing into the nephrons. This occurs in the human when the plasma level exceeds 10 mM. Glucose is then present in the urine and causes an osmotic diuresis. Insulin lowers blood glucose levels by increasing its uptake into muscle and adipose tissue and also by promoting glycogen formation from glucose. Another of the effects of insulin is to decrease fatty acid release from adipose tissue. The inability to utilize glucose along with the increased release of fatty acids from fat stores results in a loss of weight.

Weight-loss was observed in some rats involved in this study after induction of diabetes (see section 3.3.1). It was, therefore, considered

advisable to record the weights of the animals each week in order to follow their progress and give insulin where necessary (see section 3.2.2).

Different authors recommend different dosages of STZ : Sharma and Thomas (1975), 80 mg kg^{-1} body weight; Yoshida *et al.* (1987), 65 mg kg^{-1}; Ekstrom and Tomlinson, (1990), 50 mg kg^{-1}; Bisby, (1980), 50-75 mg kg^{-1}. Personal discussion with Dr. R. Malik (Aberdeen University) whose own experiments had involved the use of doses of 60 mg kg^{-1}, resulted in the decision to use the same amount in the initial experiments of this study. However, a severe loss of weight in some cases (see section 3.2.2) and the presence of ascites prompted a reduction in the dose which was administered subsequently. It was found that a dose of 55 mg kg^{-1} was sufficient to fulfil the criteria set in terms of blood sugar level (see below) yet did not cause a harmful catabolic response. As the objective of STZ administration was to produce hyperglycaemia within a chosen range, and given the fact that individual rats responded differently to the same dosage of STZ in terms of the raising of the blood glucose level (see below): the difference in the amount of STZ given to different groups of rats was not considered to be of consequence. Bisby (1980) administered STZ in a range of 50-75 mg kg^{-1}.

Preparation of STZ in citrate buffer.

Sterile water was added to a mixture of 1 g of STZ and 220 mg of citric acid to give 10 ml of solution, then 0.1 M sodium hydroxide was added to give a pH of between 3.5 and 4.5. The 10 ml solution was diluted to 30% by the addition of distilled water to give 23 ml.

Total amount of fluid required = 100/ 30 x 10 = 33 ml

10 ml are already present,

therefore, 23 ml are needed to dilute to 30%.

Each ml of solution then contained:

30 mg of STZ,

6.67 mg of citric acid.

STZ is stored at a temperature below 0°C because of its breakdown at higher temperatures. For the same reason the prepared solution had to be used within 8 hours of preparation.

The amount of the solution to be injected was calculated as follows:

Amount of STZ given to each animal = 55 mg kg^{-1}

Therefore, amount of STZ required for each animal

= (weight of animal x 55)/ 1000

Each ml of prepared solution contains 30 mg STZ

Therefore, volume of solution injected

= (amount of STZ required)/ 30

The required amount of solution, as calculated above, was drawn up into a syringe and the needle was inserted into the animal's abdomen at the linea alba and the plunger drawn out; if no blood, urine or faecal matter was drawn up into the syringe the solution was delivered intraperitoneally. It was important to determine the absence of blood, urine or faeces in the syringe as this indicated that the needle had not been inserted into a blood vessel,

bladder or intestine and ensured deliverance of STZ solution into the peritoneal cavity.

A solution was prepared containing similar amounts of citric acid and sodium hydroxide but the STZ was omitted. Control animals were injected intraperitoneally with the solution at the same time as the other rats received the STZ solution.

Monitoring of blood sugar levels.

Blood glucose levels were monitored using the 'Reflolux' S reflectance photometer in conjunction with BM-Glukotest 1-44 test strips (both: Boehringer Corporation (London) Ltd, U.K.). The Reflolux meter can determine blood glucose in the range of 0.5 to 27.7 mM (10 to 500 mg dl^{-1}). To verify that the Reflolux photometer was giving accurate readings of blood glucose, a calibration curve was plotted of standard glucose solutions against the Reflolux readings. The relationship between the two sets of values was almost linear proving the Reflolux to be very accurate (see appendix 2).

Blood glucose levels were measured the day after injection of the STZ solution and then on the two consecutive days, as blood glucose levels have been found to reach a peak 1 to 2 days after STZ administration (Like & Rossini, 1976). Blood glucose values were subsequently checked at weekly intervals and after this method of verifying that a continual diabetic state had been established, blood glucose levels were checked every third week until death to ensure that the required levels were maintained. Checks were considered necessary in case β cell regeneration occured to any extent and insulin production became adequate to raise blood glucose to normal levels. However, Brosky and Logothetopoulos (1969) noted that the regenerative capacity of β cells surviving cytotoxic injury resulting from STZ

injection was limited, and guinea pigs failed to recover over a nine month period. Also, the work of Junod *et al.* (1967) in rats led them to conclude that STZ has a rapid and irreversible cytotoxic effect on pancreatic β cells. In the experiments reported here, glucose levels always remained high and additional injections of STZ were never required.

In the human, normal blood glucose is 3.5 to 8 mM; diabetes is diagnosed if there is high random blood glucose after a 10 hour fast of 15 mM. In their experiments with STZ induced diabetic rats, Ekström *et al.* (1989) chose 13 mM as the cut off point for diabetes. 14 mM was chosen as the minimum blood sugar level to indicate diabetes in the experiments described here.

2.3 Anaesthesia.

Before commencing any of the electrophysiological work and before exposing the sciatic nerve for either crushing, suturing or grafting, all rats were anaesthetized in one of two ways described below.

Inhalational anaesthesia was only used to anaesthetize those rats used in the experiments presented in chapter 7 that were subsequently decerebrated (the reasons for using this method are described in chapter 7). All other animals were rendered unconscious using an intra-muscular injection of a mixture of a neuroleptanalgesic and a benzodiazepine.

Animals were considered adequately anaesthetized at the point when the response to various tests were abolished:

(1) Flexor withdrawal reflex. The rat does not respond to nociceptive stimuli, by failing to withdraw its foot after it is pinched.

(2) Corneal blinking reflex. A blinking reflex is not initiated when the rat's eye is touched with a cotton bud.

(3) Auditory reflex. The animal does not respond by general muscular contraction to an auditory stimulus, when a bunch of keys is dropped beside the rat.

2.3.1 Intra-muscular injection of analgesic.

The rats were injected intra-muscularly with 0.5 ml kg^{-1} of a 1:1 mixture, by volume, of a solution of Hypnorm (fentanyl citrate, 0.315 mg ml^{-1} and fluanisone, 10 mg ml^{-1}; Janssen Pharmaceuticals Ltd) and Hypnovel (midazolam hydrochloride, 5 mg ml^{-1}; Roche Products Ltd).

Although not used in humans as an anaesthetic, a hypnorm/hypnovel mixture is commonly used in animal experimentation to render the animal unaware of and unresponsive to, painful stimulation.

Hypnorm contains fentanyl citrate which is an opioid and morphine-like drug of the phenylpiperidine series. Morphine-like drugs have their greatest effects in the substantia gelatinosa, where opioid receptors are numerous. The drugs act by producing an inhibitory effect on nervous transmission from nociceptive afferents. Fentanyl is commonly used as an adjunct to anaesthesia in humans (Rang & Dale, 1987).

Hypnovel contains midazolam hydrochloride which is a benzodiazepine with sedative and anxiolytic properties. Benzodiazepines specifically augment the actions of gamma-amino-butyric acid (GABA). Two distinct binding sites exist for benzodiazepines and GABA, although the former are not present in peripheral neurons. Benzodiazepines, therefore, act mainly at receptors located in the cerebral cortex, limbic system and mid-brain (Rang & Dale, 1987). Midazolam is used in humans as a premedicating agent and in animals it has a marked 'taming' effect as well as producing a decrease in muscle tone.

The anaesthetic was administered, in the amounts described above, into the gluteal muscles of the right limb.

2.3.2 Inhalational anaesthesia.

For reasons explained in chapter 7.2.1, some rats were anaesthetized with a gaseous mixture of halothane (Fluothane, R.M.B. Animal Health Ltd, Dagenham, U.K.), and nitrous oxide and oxygen (in a 1:1 mixture), prior to decerebration. This gaseous mixture is commonly used in human general anaesthesia; the advantage being that although the mixture must be given continuously, when the anaesthetic is withdrawn, the effects 'wear off' very rapidly. Halothane is a very potent anaesthetic, its minimal alveolar concentration (MAC) being only 0.8%. However, halothane is a respiratory and cardiovascular depressant. Nitrous oxide is not a very potent anaesthetic at the concentrations used when it is, necessarily, combined with oxygen, however, it does have an analgesic effect at concentrations too low to cause unconsciousness. The major advantage in the use of nitrous oxide is that it lowers the MAC of halothane so that unconsciousness is obtained at concentrations less likely to cause respiratory and cardiovascular complications.

Induction of anaesthesia.

The animal to be anaesthetized was placed in a specially designed, small animal, anaesthetic chamber with an inflow and an outflow. The inflow was connected via plastic tubing to a modified Boyle's anaesthetic machine carrying cylinders of oxygen and nitrous oxide, also a temperature regulated vaporizer for halothane. Such a special vaporization chamber is important in order to control the concentration of halothane very precisely. The outflow was connected to a plastic container filled with soda-lime. A 4% mixture of halothane in a 1:1 mixture of nitrous oxide and oxygen was passed into the

box. When the animal became unconscious, it was removed and placed on the operating table. The anaesthetic machine was reconnected to the animal via plastic tubing and a purpose-built mask (made from a cut-off 50 ml syringe). The mask was placed loosely over the rat's nose and mouth, to allow the inhalation of anaesthetic but also the exit of expired gases. When the animal was deeply anaesthetized (see tests above), the concentration of halothane was turned down to 1.5%, in a 1:1 mixture of nitrous oxide and oxygen, as before. This amount was sufficient to maintain the depth of anaesthesia required to commence operating.

Tracheostomy and ventilation.

In the decerebration experiments it was necessary to ventilate the lungs to supply oxygen in order to maintain consistent values of arterial blood gases (although these were not measured). A tracheostomy was performed and the rat was connected to a pressure cycled ventilator (Harvard Apparatus Ltd, Edenbridge, U.K.):

Using a scalpel, a vertical incision was made extending, approximately 2.5 cm, rostrally from the manubrium sterni. The skin was retracted and the underlying connective and glandular tissue was removed to expose the strap muscles. These were separated in the midline and retracted laterally to expose the trachea. Two lengths of 4/0 Mersilk (Ethicon Ltd, Suture Division, Edinburgh, U.K.) were passed, using an aneurysm needle, around the trachea and a transverse incision was made through its anterior half, between adjacent cartilaginous rings. A short metal endotracheal tube attached to an inspiratory/expiratory Y-piece was inserted into the trachea and ligated in place using the 4/0 silk ties. The inspiratory arm of the Y-piece was connected to the outflow port of the ventilator. The expiratory arm was connected to the return port of the

ventilator and ultimately to a waste gas collector system. The level of halothane was decreased to 0.5% in oxygen and nitrous oxide, as described above. The ventilator was set to deliver the anaesthetic mixture at a constant pressure of 70 cm H_2O at a rate of 100 min^{-1}.

2.4 Exposure of the Sciatic Nerve.

The animals were anaesthetized as in 2.3. The left leg was shaved using electrically operated clippers (Oster Professional Products, Milwaukee, U.S.A.) and cleaned with a solution of chlorhexidine 0.5% in 70% alcohol.

An incision was made through the skin on the lateral aspect of the left thigh, midway along a line between the hip and knee joints. Retraction of the skin exposed the caput vertebralis part of the biceps femoris and also the gluteus superficialis. The aforementioned muscles were separated along the plane dividing them and the biceps femoris was retracted. This exposed the sciatic nerve lying deep to the biceps femoris and superficial to the femur.

2.5 Experimental Groups of Animals.

For diabetic and non-diabetic cohorts of animals, 5 experimental groups were set up. Each experiment, therefore, contained 2 sets of 5 groups of animals i.e. 10 groups *in toto*. Each group of animals, as defined by the treatment of the sciatic nerve, contained a minimum of 5 rats. The 5 types of treatment of the sciatic nerve are listed below:

1) Untreated (control) group.

2) Crush injury to the sciatic nerve (nerve crush): this produced a Sunderland type II injury (axonotmesis).

3) Transection of the sciatic nerve and repair by epineurial suture (nerve-to-nerve suture): Sunderland type V injury (neurotmesis).

4) Transection of the sciatic nerve and repair with a full thickness nerve autograft (nerve graft): Sunderland type V injury (neurotmesis).

5) Transection of the sciatic nerve and repair with a coaxial, freeze-thawed, autologous muscle graft (muscle graft): Sunderland type V injury (neurotmesis).

2.6 Treatment of the Sciatic Nerve.

The sciatic nerve was exposed as in 2.4. As stated in 2.5, the procedures described below were performed on a minimum of 5 diabetic and 5 non-diabetic rats in each experiment.

Nerve Crush.

The sciatic nerve was freed from the surrounding tissue bed, enough to insert the blades of watchmakers' forceps under the nerve, using microsurgical instruments. Minimal disruption of the blood supply to the nerve was, therefore, achieved. An operating microscope (Weck Fibermatic 0902A1, Long Island City, New York) was used to assist vision throughout. Haemostasis was obtained by means of a Codman Malis bipolar coagulator (Codman & Shurtleff Inc, Randolph, Massachussettes, U.S.A.). High frequency A.C. current passing between the tips of the diathermy forceps induces heat in the adjacent tissue, causing localized coagulation of protein and hence, sealing of vessels.

The nerve was crushed between the smooth jaws of a microsurgical needle holder (Codman, Randolph, Massachussettes, U.S.A.) at a point approximately 1 cm distal to the emergence of the nerve from the sciatic

notch. Using the ratchet mechanism of the needle holder, the jaws were locked into the second position of the ratchet for 10 s. A similar and reproducible axonotmesis was thereby produced in each nerve, of the same time and the same pressure. Plate 2.1 is a photograph of a rat sciatic nerve that had been crushed for 10 s between the smooth jaws of a microsurgical needle holder.

The wound was closed as described in section 2.7.

Transection of the sciatic nerve and repair by epineurial suture (n-n suture).

The sciatic nerve was freed from the surrounding tissue bed as above. The disruption of blood vessels was necessarily greater in the neurotmesis type injuries and subsequent repair, as the nerve had to be freed to a greater extent in order to operate without tension. Haemostasis was obtained in the same way as described for the crush injuries.

The nerve was divided at a distance of 1 cm from the point where the sciatic nerve emerges from the sciatic notch. The epineurium was clearly seen when the nerve was viewed under the operating microscope. Using 10/0 polyamide sutures (Ethilon, Ethicon Ltd, U.K.), the epineuria of the two nerve stumps were sutured together; 4 to 6 interrupted stitches were used. Additional stitches were used where necessary to prevent fascicles from escaping the suture line.

The muscle and skin layers were closed as described in 2.7.

Transection of the sciatic nerve and repair with a full thickness nerve autograft (nerve graft).

A full thickness nerve autograft involved the removal of a 1 cm length of sciatic nerve and suturing the excised portion back in place.

The sciatic nerve was exposed and freed as described above. The nerve was divided at a point of 0.5 cm from the emergence of the sciatic

nerve from the sciatic notch. The epineuria of each of the two stumps were sutured together using 4-6 10/0 polyamide sutures. Although the sciatic nerve is not monofascicular, no attempt was made to align the fascicles. The nerve was divided again 1 cm distal to the suture line. The nerve stumps were sutured together in the same way. Plate 2.2 is a photograph of a rat sciatic nerve that had been repaired with a full thickness nerve autograft.

The muscle and skin were sutured together as described in 2.7.

Transection of the sciatic nerve and repair with a coaxial, freeze-thawed, autologous muscle graft.

An incision was made through the skin on the lateral aspect of the left thigh to expose the biceps femoris, as described in 2.4. After detaching the muscle from its origin, a portion of the biceps femoris, of about 2 cm by 0.5 cm by 0.5 cm, was removed with the long length in parallel with the muscle fibres. The freezing-thawing procedure described below causes a shrinkage of the muscle of up to 50% of its original size (Glasby *et al.*, 1992); it is for this reason that a larger piece of muscle, than is necessary for the graft itself, was removed.

The excised portion of muscle was pinned to a small piece of card and then immersed in liquid nitrogen. Immediately after immersion there was bubbling, due to the evaporation of the liquid nitrogen on contact with the warm tissue. When the tissue reached the same temperature as the nitrogen, the bubbling stopped. At this point the tissue was removed and put into a beaker of distilled water. While the muscle was thawing the sciatic nerve was freed from its tissue bed and divided at a distance of 0.5 cm distal from the emergence of the nerve from the sciatic notch. The nerve was divided again, 1 cm distal to the first transection, and the piece of sciatic nerve was removed. When completely thawed the muscle was removed from the distilled water and cut to size using a scalpel. The muscle was cut

to approximately 1 cm by 0.2 cm by 0.2 cm with the fibres running along the long axis of the muscle block. The epineurium of each sciatic nerve stump was sutured to either end of the muscle block, using 4-6 10/0 polyamide stutures. Plate 2.3 is a photograph of a rat sciatic nerve that had been repaired with a freeze-thawed skeletal muscle autograft.

The cut side of the biceps femoris was sutured to the gluteus superficialis using interrupted 6/0 vicryl sutures. The skin was then sutured as described in 2.7.

2.7 Closure of the Wound.

To close the wound the biceps femoris was sutured to the gluteus superficialis using interrupted 6/0 Vicryl sutures (Ethicon Ltd, U.K.). The skin was sutured using 3/0 Vicryl (Ethicon Ltd, U.K.). The wound was again cleaned with chlorhexidine in alcohol and the rat was left to recover under observation.

After operating on the sciatic nerve, the rats involved and the unoperated control animals were maintained in the Medical Faculty Animal Area, in the way described in 2.1, for a period of 150 days.

2.8 Termination of Experiments.

After 150 days had elapsed, the rats were anaesthetized as in section 2.3 in preparation for electrophysiological study. At the end of each experiment the animals were painlessly killed using one of the methods scheduled in the Animal Scientific Procedures Act, 1986. The methods employed were either cervical dislocation or a lethal dose of Lethobarb (phenobarbitone, 20% w/v, J.M.Loveridge PLC, Duphar Veterinary Ltd, Southampton, U.K.) administered intraperitoneally.

2.9 Preparation of Nerve for Histological Examination.

Nerve was required for histological examination from those animals used in the work presented in chapters 4 and 8. The animals were anaesthetized as in section 2.3 and nerve was removed.

Tissue fixation.

The aim of tissue fixation is to prevent autolysis and preserve the tissue, in a state as close as is possible, to that existing in life. Ideally there should be no shrinkage or swelling; the fixative should penetrate rapidly and evenly; it should harden the tissue and render it receptive to staining. Glutaraldehyde has become the standard fixative for electron microscopy. Although the molecules are larger than formaldehyde molecules and therefore penetrate tissue more slowly, the specimens used in electron microscopy are small and the most important consideration is the effectiveness of the fixative. Glutaraldehyde is a better fixative than formaldehyde and its rapid fixing action maintains ultrastructure much better (Drury & Wallington, 1980). This is because it contains two more 'reactive' aldehyde groups in its structure (as opposed to formaldeyde's one, less 'reactive' group), which cross link the basic groups of amino acid residues (lysine), found on the outside of protein. Glutaraldehyde fixative is generally followed by treatment with osmium tetroxide (OsO_4) and this was the combination used in the work described below. OsO_4 reacts with double bonds of hydrocarbon side chains of lipid to give an osmate ester and hence, fat is rendered insoluble. This step is of particular importance in fixing of peripheral nerve because of the need to preserve the fatty myelin sheaths.

Specimens of nerve from experimental animals were removed in order to produce 0.5-0.7 μm semithin sections for morphometric analysis. Lengths of approximately 0.5 cm of peroneal nerve were removed, as described in chapter 4.2.7, and laid onto small pieces of labelled card, without stretching the nerve. The card and nerve were then put into a test tube containing 4% glutaraldehyde in 0.1 M cacodylate buffer (see appendix 3) to harden the tissue. After an hour the nerve was removed from the test tube and cut transversely, under a dissecting microscope (Leitz Wetzlar, Germany), into pieces of 1 mm in length. It is necessary to cut the nerve into pieces in order to allow penetration of fixative throughout the specimen; glutaraldehyde has the disadvantage of penetrating poorly into large tissue blocks (Drury & Wallington, 1980). The nerve was left for a further hour in test tubes of glutaraldehyde fixative before replacing the fixative with 5% sucrose in cacodylate buffer (appendix 4). At this point the tissue was left overnight in buffer.

The tissue was then post-fixed in 1% OsO_4 in 0.1 M cacodylate buffer for 2 hours (appendix 4).

Tissue processing.

Fixed tissue is too soft to cut and therefore has to be supported in an embedding medium. The thinner the sections of tissue required, the harder the embedding medium has to be. Araldite is an epoxy resin and is the medium used for electron microscopy because unlike acrylic resins, it is stable in an electron beam. Araldite is immiscible with water therefore various stages of dehydration must be performed in order to allow penetration of the embedding medium through the tissue.

The tissue was processed as follows:

(1) After osmication the tissue was washed in sucrose buffer.

(2) The specimens were dehydrated in 10% alcohol for 10 minutes.

(3) They were then dehydrated in 70% alcohol for 30 minutes.

(4) They were further dehydrated in 80% alcohol for 30 minutes.

(5) Dehydrated in 90% alcohol for 30 minutes.

(6) Dehydrated in 3 changes of 100% alcohol, each for 30 minutes.

(7) Alcohol is immiscible with Araldite. Propylene oxide is miscible with both alcohol and Araldite and is, therefore, used as an intermediate change before the tissue can be embedded in Araldite. The tissue was placed in propylene oxide for 2 changes of 30 minutes.

(8) The specimens were placed in a mixture of propylene oxide: Araldite (1:1) for 30 minutes.

(9) The tissue was then placed in a mixture of propylene oxide: Araldite (1:2) for 30 minutes.

(10) The tissue was placed in Araldite at 40^{o}C for 1 hour.

(11) The Araldite was replaced and left overnight at room temperature to allow time for the tissue to been penetrated by the Araldite.

Stages 4 to 10 were performed by a Lynx *el* microscopy tissue processor (Australian Biomedical Corporation Ltd, Victoria, Australia). The tissue was removed from the processor and orientated, with the aid of a dissecting microscope, in moulds containing liquid Araldite. Araldite in the liquid state is a monomer and has to be polymerized to make it hard. The Araldite was polymerized by heating at 60°C in an oven for 48 hours.

Sectioning.

The Araldite blocks were removed from the moulds and trimmed using a razor to expose the transverse face of the nerve tissue. Glass knives were made using a LKB knifemaker (LKB Produkter, Stockholm, Sweden). The knives were placed in a Reichert OMU3 ultramicrotome (Reichert viz. Leica, Cambridge, U.K.) and semithin sections of 0.5-0.7 μm thickness were cut. The sections were laid on drops of water on glass slides and dried on a thermostat hotplate. Drops of toluidine blue were placed over the sections and they were stained for 10-30 s. The stain was then washed off with tap water and the sections were left to dry.

2.10 Processing for Wax Histology.

Wax histology was used on pancreatic tissue (chapter 3) so that immunocytochemistry could be performed on the prepared tissue sections. Wax was chosen because it can easily be removed from tissue sections and allow subsequent penetration of the antibodies used in the immunocytochemical staining process.

Tissue fixation.

Formaldehyde is the most commonly used fixative in wax histology and was used in this work because of the small size of the formaldehyde molecules which allow rapid and great depth of penetration.

Fresh pancreas was removed from the animal (see chapter 3.2.5) and placed in a beaker containing 4% formal saline (10 ml of 40% formaldehyde in 90 ml of 10% saline), and left for 48 hours. After the tissue was fixed, it was stored in 70% alcohol and left for 24 hours.

Tissue processing.

The tissue was processed as follows:

(1) Dehydrated in 80% alcohol for 30 minutes.

(2) Dehydrated in 90% alcohol for 30 minutes.

(3) Dehydrated in 2 separate aliquots of 96% alcohol for 30 minutes each.

(4) Dehydrated in 2 separate aliquots of absolute alcohol for 30 minutes each.

(5) Cleared in xylene for approximately 15 minutes. Clearing agents are miscible with both alcohol and wax; clearing is therefore the essential step between dehydration and embedding. Xylene was used because in contrast to other clearing agents such as inhibisol, it is possible to observe the end point when the tissue becomes transparent i.e. the tissue has been 'cleared'. Clearing tends to make the tissue very brittle.

(6) After clearing, the tissue was embedded in wax (Paraplast; Monoject Scientific Incorporated, Athy, Ireland) at 62^{o}C. Embedding

was carried out in a vacuum because this causes air to leave the tissue and speeds up the rate of penetration of wax into the tissue. The specimen was placed in a glass jar containing wax and this jar was placed in a vacuum embedder heated to 62 ^{o}C. The wax initially solidified around the tissue as the tissue was cooler than the wax. The tissue was left until the solidified wax melted again. At this point of thermal equilibrium the wax and specimen had reached the same temperature. A vacuum was produced in the sealed chamber using a manually operated pump attached to the chamber and the tissue was left for 20 minutes. Bubbles of air were observed leaving the tissue as penetration occurred.

(7) The tissue was placed in a glass jar containing fresh wax at 62^{o}C and procedure (6) was repeated.

(8) Procedure (7) was repeated.

The tissue was removed and orientated in moulds containing fresh hot wax. Blowing the top surface of the wax in the moulds caused it to solidify, now the moulds were placed in a bath of cold water until all the wax was solid.

Sectioning.

Sections of approximately 7 μm were cut using a Reichert-Young 2050 microtome (Leica, Cambridge, U.K.). The sections were laid out onto drops of water on TESPA (3-aminopropyltriethoxyxylane) coated slides (appendix 5). Coating of the slides allows adhesion of the sections to the slides. The slides were warmed on a hot plate; as the wax warmed the

sections could be teased out until they lay flat. The excess water was drained onto tissue paper and the sections were dried in an oven at 37°C.

Plate 2.1 A photograph of a rat sciatic nerve that had been crushed for 10 s between the smooth jaws of a microsurgical needle holder

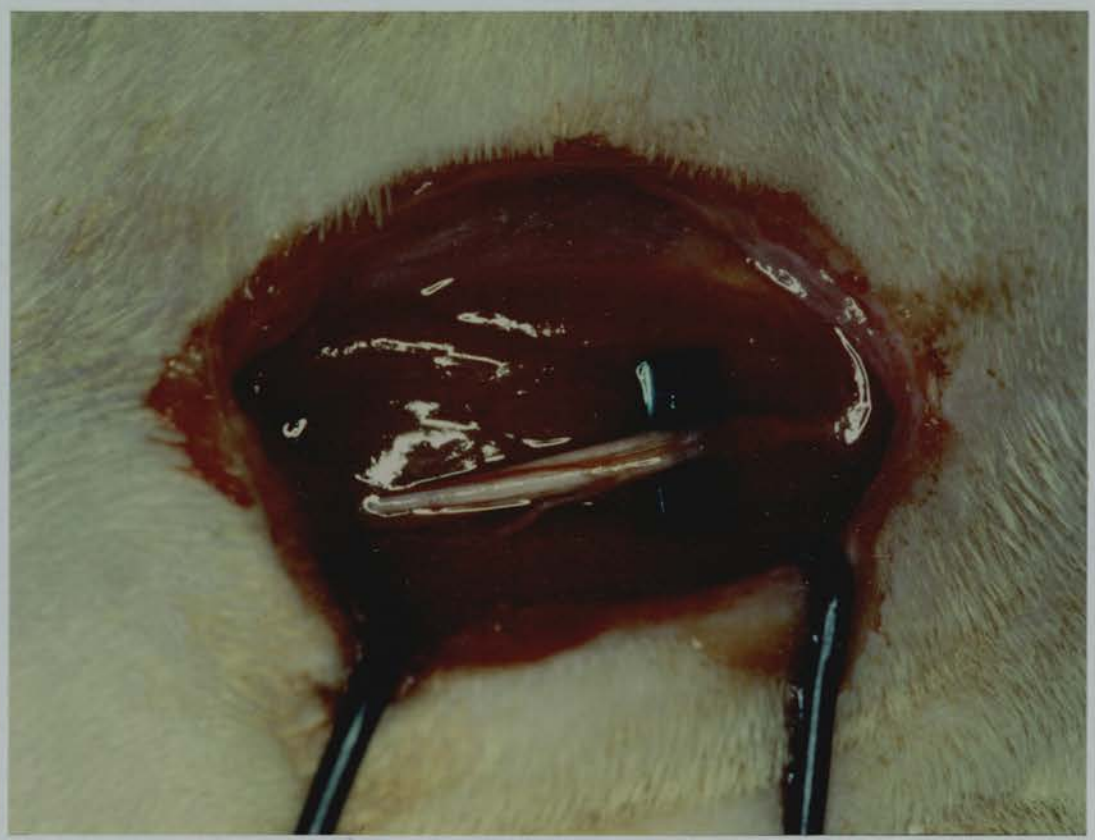


Plate 2.2 A photograph of a rat sciatic nerve that had been repaired with a full thickness nerve autograft

Plate 2.3 A photograph of a rat sciatic nerve that had been repaired with a freeze-thawed skeletal muscle autograft

CHAPTER 3

Verification of Diabetic Pathology

3.1 Introduction.

3.1.1 Aims.

The work presented in this chapter was designed to provide evidence that a metabolic state, similar to that of diabetes mellitus in humans, had been established in a population of rats that had been given a single dose of Streptozotocin (STZ). Further, that the diabetic state was maintained for the duration of the experiments on nerve regeneration (i.e. 150 days). Changes in the β cells of the pancreatic islets of Langerhans, levels of whole-blood glucose, changes in body weight and development of cataracts were all monitored.

3.1.2. The Use of Streptozotocin in Experimental Diabetes.

Before the use of diabetogenic agents, the diabetic state was artificially induced in animals by partial or total pancreatectomy. In more recent years, the most effective compound used to produce experimental diabetes was alloxan; however, more recently the use of alloxan has lost favour since the discovery by Rakieten *et al.* in 1963 of a broad spectrum antibiotic (and anti-tumour drug), STZ, that was found to be diabetogenic in dogs and rats. STZ is a by-product of the bacterium Streptomyces achromogenes. Junod, Lambert, Orci, Pictet, Gonet and Renold (1967) found that STZ had a very specific action on pancreatic β cells of rats and they suggested that the reason why STZ was less toxic than alloxan, especially in respect of the kidney, was this specificity of action. These authors found some evidence of focal hepatic necrosis in rats between 2 and 36 hours after STZ

administration but they said that any anomalies found in the liver and the kidney could be explained by the effects of hyperglycaemia and hyperlipidaemia. Junod, Lambert, Stauffacher and Renold (1969) found the extent of diabetes induced by STZ was dose dependent. Mild diabetes was induced with doses of 35 mg kg^{-1} of STZ, whereas, doses of over 100 mg kg^{-1} commonly caused severe ketosis and death between 2 and 3 days after administration of the drug. The diabetogenic and lethal effects of alloxan, however, are produced within a much smaller range of doses.

It has been mentioned in chapter 1.6 that most of the research into nerve regeneration in diabetes has been very short-term. It was one of the aims of the experiments described here to compare the end-points of regeneration in control rats with diabetic rats and necessarily, the experiments described in this thesis were longer than many previous experiments on nerve regeneration in STZ diabetic rats. Tomlinson, Gardiner, Hebden and Bennett (1992) stated that insulin supplementation has been used in some studies involving STZ diabetes such that the animals used could survive long-term experiments. To ensure that the rats used in the work presented here survived the duration of the experiments, insulin supplementation was used (see chapter 2.2).

3.1.3 The Action of Streptozotocin.

Rakieten *et al.* (1963) suggested that STZ produced a state of hyperglycaema by causing necrosis of the β cells of the pancreas. In contrast, Arison, Ciaccio, Glitzer, Cassaro and Pruss (1967) favoured a mechanism of inhibition of insulin synthesis and degranulation of β cells without cell necrosis. Junod *et al.* (1967) showed that STZ caused changes in the β cells of rat pancreas as early as 1 hour after its administration and they found massive necrosis of the β cells of the pancreatic islets 7 hours

after the administration of STZ. Remaining β cells were few and mostly degranulated. Brosky and Logothetopoulos (1969) also found that STZ produced hyperglycaemia by extensive necrosis of the β cells of the pancreas.

The mechanism behind the specific destruction of β cells has not yet been confirmed. Like and Rossini (1976) suggested that STZ acts by initiating a cell-mediated immune reaction. Evidence for this theory came from the observation that small sub-diabetogenic doses of STZ given to mice, resulted in the appearance of large numbers of lymphocytes and moderate numbers of macrophages in the pancreatic islets, at a time appropriate for an immune reaction of the above type. However, these authors also found that the islets were virtually inflammation-free in those mice that had received a single large dose of STZ and, therefore, a non-specific inflammatory response to low-grade β cell injury induced by STZ could not be excluded in the animals given multiple small doses of STZ. Brosky and Logothetopoulos (1969) also failed to observe infiltration of leukocytes into the islets of their mice and guinea pigs. Using a method of freeze-etching, Orci, Amherdt, Malaisse-Lagae, Ravazzola, Malaisse, Perrelet and Renold (1976) demonstrated ultrastructural changes in the membranes of β cells of mice after the administration of both alloxan and STZ. They suggested that the mechanism of action of both alloxan and STZ in causing β cell necrosis was to produce "ultrastructural disarray and possibly impairment of β cell membranes".

3.1.4 Changes in the Pancreas in Clinical and Experimental Streptozotocin Diabetes.

Brosky and Logothetopoulos (1969) investigated the changes in the pancreatic islets of mice and guinea pigs after the administration of STZ. In

the mouse, they saw pyknotic β cell nuclei after 3-4 hours; by 36-48 hours the islets appeared to have collapsed with only a few β cells remaining. They found that the necrotic β cells were removed without any significant inflammation. No significant changes were seen in the pancreas after 4 to 5 days and, therefore, 5 days after administering STZ was the time chosen, in the experiments presented here, for the investigation of the rat pancreases (see chapter 3.2).

Some of the changes that occur in the pancreas of animals after STZ administration are very similar to the changes that occur in humans with juvenile (type I) diabetes. Gepts (1965) investigated the changes in human pancreases removed from patients who had died shortly after being diagnosed with type I diabetes mellitus and also from patients who had survived up to 37 years after being diagnosed with type I diabetes mellitus. He found that the number of β cells in the human pancreas was reduced to less than 10% of normal numbers in patients who presented acutely with diabetes and died shortly afterwards. Beta cells of patients who suffered chronically with type I diabetes were almost completely absent. In this respect, the type of diabetes induced by STZ can be compared to both the acute and chronic forms of type I diabetes in humans. Like and Rossini (1976) found that multiple sub-diabetogenic doses of STZ in mice caused a gradual decline in numbers of β cells until few remained, whereas, large single doses of STZ in mice produced a reduction in numbers of β cells, although some large islets contained a greater number of β cells (Brosky & Logothetopoulos, 1969). Gepts (1965) found evidence of inflammation in and around the pancreatic islets of those patients who died shortly after being diagnosed with juvenile diabetes. In contrast, inflammation was not marked in mice that had been given a single dose of STZ (Like & Rossini, 1976; Brosky and Logothetopoulos). Also, Gepts (1965) did not observe

inflammatory infiltrates in the pancreatic islets of patients suffering chronically with juvenile diabetes, whereas, Like and Rossini (1976) found inflammation in the pancreases of mice that had been given sub-diabetogenic doses of STZ (see above).

Another difference between human type I diabetes and STZ induced diabetes is that neo-formation of islets has been observed by Gepts (1965) in humans who died shortly after an acute onset of the disease. Brosky and Logothetopoulos (1969), however, failed to find any evidence of proliferation of duct epithelial or centro-acinar cells. The latter authors found evidence of mitotic activity of β cells in their mice that had been injected with STZ, although Gepts (1965) found only two cells in one case of acute onset diabetes that showed mitotic activity. Brosky and Logothetopoulos (1969) observed that their STZ diabetic mice showed no reversion from the diabetic state and Gepts (1965) suggested that the failure of humans to recover from diabetes may be because the capacity of human pancreatic parenchyma to form new islets becomes exhausted by the time of onset of the disease. Similar to STZ induced experimental diabetes, Gepts (1965) said that the changes seen in human pancreatic tissue is evidence that juvenile diabetes is not caused by an inadequacy of the pancreas to function normally but is rather, evidence of a "progressive deterioration under the influence of an extrapancreatic factor".

3.1.5 Body Weight Changes in Diabetes.

The presenting symptoms of diabetes mellitus include: polydipsia, polyphagia and polyuria. Deficiencies of insulin and/or insulin resistance of tissues affects the normal biochemistry of the body in a number of ways. The entry of glucose into the cells that require insulin for its uptake is drastically reduced. In order to obtain glucose for entry into the glycolytic

pathway, liver glycogen is broken down to glucose. Free fatty acids are mobilized from peripheral stores as a result of lack of insulin and they are circulated to the liver, where they are oxidized to acetoacetylCoA and acetylCoA. AcetylCoA can then enter the Krebs' cycle for production of energy; alternatively, both acetylCoA and acetoacetylCoA can be converted to ketone bodies. The net effect in diabetes mellitus is one of gluconeogenesis and mobilization of free fatty acids. The resultant hyperglycaemia and production of excess ketone bodies leads to hyperosmolarity of the blood and acidosis.

Similar biochemical changes occur in animals injected with STZ as in patients with diabetes mellitus. Ketosis is most common in type I diabetes in humans and in this respect, STZ diabetes is more like type II diabetes. This is because ketonuria has been reported to be rare in rats that have been injected with STZ (Junod *et al.*, 1967). However, polydipsia, polyphagia and poluria are encountered in STZ diabetic animals (see chapter 2.2). Junod *et al.* (1967) administered a dose of STZ of 65 mg kg^{-1} to male Wistar rats and found that their body weight remained, on the average, at or near the initial value. Their control animals, which were at a similar stage of development as the diabetic animals, gained 30-40 gwt each week. These authors also noticed that lipaemia was quite pronounced in some cases of STZ diabetes and that abdominal body fat stores, which were reduced after a week, had totally disappeared after a month.

3.1.6 Development of Cataracts in Diabetes.

The development of cataracts in animals with hyperglycaemia is common (Jakobsen *et al.*, 1981). Cataracts arise as a result of osmotic changes in the lens which causes refractive errors.

3.2 Materials and Methods.

3.2.1 Preparation of Experimental Animals.

Brosky and Logethoutopoulos (1969) found that STZ administration causes permanent diabetes and hyperglycaemia in mice and guinea pigs; Junod *et al.* (1967) confirmed these findings in rats. In order to corroborate the work of these authors, and to leave no doubt that a state of permanent diabetes had been induced in the rats used in the experiments presented in this thesis, immuncytochemical staining of the β cells of the pancreas was performed. Such staining of the β cells (as described below) was carried out on animals 5 days and 150 days after STZ administration.

10 rats were injected with STZ in citrate buffer in order to induce diabetes (chapter 2.2). A further 10 rats were injected with citrate buffer as described in chapter 2.2. 2 days later, blood glucose levels were measured using a Reflolux S reflectance photometer and BM-Glukotest 1-44 test strips (Both Boehringer Corporation (London) Ltd, U.K.).

5 days after administering the injections, 5 of the animals that had been injected with STZ in citrate buffer and 5 of the animals that had been injected with citrate buffer only, were anaesthetized as described in chapter 2.3, in preparation for the removal of the pancreas (chapter 3.2.5). The remaining 10 rats were returned to the Medical Faculty Animal Area and kept in the way described in chapter 2.1 for 150 days. After this period of time, the remaining 10 rats were anaesthetized as described in chapter 2.3 and the pancreases were removed (chapter 3.2.5).

The monitoring of rat weights and blood sugar levels described in this chapter was carried out on all of the diabetic rats used in the experiments presented in this thesis.

3.2.2 Monitoring of Rat Body Weights.

Owing to the inability of diabetic patients to utilize glucose and to the increase in release of fatty acids from fat stores, sufferers of the disease often experience a loss of weight. Junod *et al.* (1967) reported that the body weight of rats injected with STZ remained, on average, at or near to the initial values; however, age-matched control animals gained approximately 30-40 gwt weekly. The diabetic animals were weighed weekly.

A loss in weight indicates an inability of the animal to obtain sufficient energy from its food and will, therefore, jeopardize longevity. The fluctuations of individual rat weights were monitored closely because the survival of the rats over the duration of the experiments (i.e. 150 days) was of paramount importance. Those animals whose weight fell by more than 50 gwt from their initial weight or whose weight fell below 350 gwt were injected with insulin (see below).

The final weights of the non-diabetic animals were also noted so that a comparison could be made between diabetic and non-diabetic animals at 150 days. The final weight is an important consideration, particularly in the work presented in chapter 5 when recovery of receptive field area was measured. Bigger rats have larger receptive field areas. Size of the animal is also of importance in the work of chapter 6, where recovery of the force of contraction of the soleus muscle was measured. Bigger animals are also likely to have larger muscles and produce larger forces of contraction.

3.2.3 Monitoring of Blood Glucose.

The blood glucose levels were monitored using a Reflolux S reflectance photometer in conjunction with BM-Glukotest 1-44 test strips (both Boehringer Corporation (London) Ltd, U.K.). Blood was obtained from the tail of each rat by piercing the latter with a needle. A drop of blood was left on each strip for 1 minute before it was wiped off with cotton wool. The strip was then placed in the photometer for a further minute, at which point a reading of blood glucose level, in mM, was displayed. To check the accuracy and calibrate the photometer, standard glucose solutions were made up from pure glucose in distilled water; test strips were left in the solutions for a minute before being put into the photometer. A graph was plotted of true glucose concentration against photometer reading (appendix 2).

Blood sugar levels were monitored at intervals of 3 weeks and when considered necessary (see section 3.2.4).

3.2.4 Administration of Insulin Injections.

Insulin was given to those animals that suffered a loss of weight as described in section 3.2.2. Initially, a single dose of 5 units of bovine insulin (Hypurin, protamine zinc insulin injection BP, 100 $i.u.ml^{-1}$, CP Pharmaceuticals Ltd, Wrexham, U.K.), given weekly, was injected subcutaneously. Blood sugar was tested, as described in section 3.2.3, 4 days after the insulin injection. If the animal continued to lose weight, the insulin dosage was increased to 5 units given twice weekly. If the animal began to put on body mass but the blood sugar levels fell below 14 mM, the insulin dosage was reduced accordingly. Insulin was, therefore, given to the rats to allow them to utilize enough glucose to survive.

3.2.5 Removal of the Pancreas.

The pancreas was removed from a total of 20 animals; 10 at 5 days and 10 at 150 days after administration of either STZ in citrate buffer or citrate buffer only. The abdominal cavity was opened through a transverse incision through skin and muscle in the transpyloric plane. The small intestine was retracted to expose the stomach and duodenum. The pancreas was visible as a diffuse mass of cream coloured tissue, extending from the c-shaped second part of the duodenum to the spleen. The whole pancreas was dissected free of its attachments to surrounding tissue, removed and placed in a container of 4% formal saline.

3.2.6 Indirect Immunoperoxidase Method for Monoclonal Antibodies.

Immunocytochemical staining was performed on sections of normal and diabetic pancreatic tissue in order to demonstrate that the STZ had produced a diminished number of pancreatic β cells. The method used was the indirect immunoperoxidase method for monoclonal antibodies. Guinea pig antibodies raised against insulin were used to stain specifically for β cells in the tissue sections after the embedding material (wax) had been removed (see section 2.9 for preparation of sections in wax).

An indirect method involves the use of a low concentration of a selective antibody which binds to a target molecule. This is followed by the application of a labelled antibody which binds to the first antibody in larger quantities. The larger numbers of labelled molecules which bind indirectly to one target molecule increases the sensitivity of the assay (Polak, 1986). Peroxidase is a frequently used enzymatic label, developed with the diaminobenzidine (DAB)-hydrogen peroxide reaction of Graham and Karnovsky (1966). Peroxidase is a coenzyme containing a redox enzyme

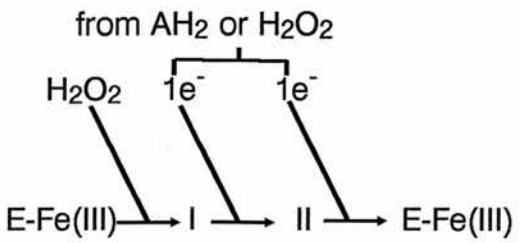
occuring naturally in red blood cells, spleen, liver and macrophages. It can be bound to protein (including antibody) by the use of glutaraldehyde. The reactions catalysed by peroxidase involve the transfer of hydrogen or electrons from a donor to hydrogen peroxide (H_2O_2), thereby oxidizing the donor (or substrate). In this experiment the substrate is DAB, the oxidation of which results in a coloured product.

The general reaction is shown in the equation below:

$$H_2O_2 + AH_2 \longrightarrow 2H_2O + A$$

where: A = the donor or substrate molecule,
which in this experiment is DAB

During the reaction the enzyme undergoes changes. The dark brown enzyme first turns olive green (compound I) in the presence of a slight excess of H_2O_2, then pale red as it forms compound II. Compound II then reacts slowly with substrate AH_2 (or with another H_2O_2 molecule) to regenerate the original enzyme (Metzler, 1977). This sequence of reactions is shown below:



where: E-Fe(III) = enzyme with the iron molecule in +3 state.

I = compound 1

II = compound 2

AH_2 = substrate (DAB)

(Metzler,1977)

Two molecules of the DAB chromogen are dehydrogenated in the process forming the blue component. In the next step, the blue component is reduced to a brownish polymer of a high molecular weight and quinoid structure. As the coloured structure formed consists of many sub-molecules of DAB, the sensitivity is increased.

A false positive result is sometimes observed in red blood cells because of the oxidation of chromogen by cell constituents. This false positive is not due to peroxidase or catalase activity in cells but rather to the activity of haemoglobin which has the same active centre as the above enzymes. A selenium-containing peroxidase is present within red blood cells but is specific for glutathione as the substrate.

Unlike peroxidase and catalase, however, haemoglobin is active in the ferrous state when it binds to oxygen (O_2). The undesirable oxidation of chromogen in the experiments presented here, by the O_2-carrying haem group of the haemoglobin molecule is prevented by the addition of H_2O_2. H_20_2 oxidizes the iron to the ferric state from the ferrous state, forming a compound methemoglobin, in which form it is unable to react with chromogen and produce a false positive.

Another spurious result, when there is generalized background staining, can be prevented by a method known as background blocking. The unwanted staining occurs when the second antibody binds indiscriminately throughout the tissue. Consequently the addition of

chromogen produces a reaction product in cells where it is undesirable. Such background staining is reduced by a method of dilution. Serum from the same species as the second antibody is applied before the second antibody itself. When the antibody is added it will be sequestered by the serum across the tissue section. This will reduce its binding to the primary antibody as well as to the rest of the tissue but, when the chromogen is added, the resulting stain will be mainly concentrated in the specific cells.

3.2.7 Tissue Processing and Immunocytochemistry.

The pancreatic tissue was processed as described in chapter 2.10. Wax sections were cut and placed on TESPA coated slides (chapter 2.10).

The indirect immunoperoxidase method for monoclonal antibodies was carried out as follows:

(a) Removal of wax and dehydration of sections:- wax was removed by immersing the slides containing the sections in xylene for 5 mins. The slides were then brought through graded alcohols, from 100% to 64%, leaving the slides for 30 s in each.

(b) Blocking of endogenous peroxidase activity:- the preparations were immersed in 3% H_2O_2 solution in methanol for 10 minutes (3 ml of H_2O_2 with 97 ml of methanol). The mechanism by which the blocking occurs is explained above (section 3.2.6).

(c) Washing of sections:- sections were rinsed in tap water for 5 minutes and then washed in tris-buffered saline (TBS) (see appendix 6) for a further 5 minutes.

(d) Background blocking:- normal serum from the species supplying the second antibody, in this case rabbit,(Sigma Chemical Company Ltd, Poole, U.K.), was pipetted over the sections. The serum consisted of 20% serum in TBS (2 ml of serum with 8 ml of TBS). After 10 minutes the excess was drained off onto a tissue.

(e) Application of monoclonal antibody:- the antibody, a guinea pig anti-porcine insulin (DAKO Ltd, High Wycombe U.K.) was diluted to 1 part of antibody in 4000 of normal rabbit serum (as made up in (d)). The solution was applied over the sections to cover them and left for 30 minutes.

(f) Washing of sections:- the sections were washed twice in TBS for 5 minutes.

(g) Background blocking:- background blocking was repeated as has been described previously in (d).

(h) Application of peroxidase conjugated with anti-immunoglobulin:- the second antibody, a rabbit anti-guineapig anti-immunoglobulin (SAPU, Law Hospital, Carluke, Scotland), was diluted to 1 in 20 of antibody in normal rabbit serum. This solution was applied to cover the sections and left for 30 minutes.

(i) Washing of sections:- the sections were washed as in (f).

(j) Application of chromogen and development of peroxidase:- 5 g of diaminobenzidine tetrahydrochloride (DAB) was dissolved in 5 ml of

DAB buffer (appendix 6) with the addition of a drop of a solution of 30% H_2O_2 in distilled water. The solution was applied to the sections and left for 3 minutes.

(k) Counterstaining of sections:- the preparations were washed in water for 5 minutes then placed in haematoxylin for about 10 s. The blue counterstain of the haematoxylin was developed in Scott's tapwater solution for 1 minute. This solution is simply an alkali solution. The sections were passed briefly through graded alcohols and then into xylene before mounting in DPX.

3.2.8 Staining with Haematoxylin and Eosin.

Sections similar to those stained by the immunocytochemical process described above were stained with haematoxylin and eosin. This staining process afforded an overall inspection of the pancreatic islets.

A drop of egg albumen (adhesive solution) was placed on each glass slide and smeared evenly over the slide. Drops of water were then placed on the slides. Wax sections cut as described in chapter 2.10, were placed on these slides. The slides were subsequently flooded with warmed water, allowing the sections to float. The slides were placed on a hot-plate and the sections were teased flat as the wax warmed. The sections were dried in an oven at 37^{o}C.

Staining was carried out on the sections as follows:

(1) Removal of wax and rehydration:- the slides were passed first through xylene and then graded alcohols to water; leaving the slides in each fluid for 2 minutes.

(2) Staining with Gill's haematoxylin:- haematoxylin is an indirect stain. Indirect stains have an intermediary called a mordant and a tissue-mordant-dye complex is formed when staining. Haematein is the chromophore of haematoxylin and in this case, potash alum is the mordant; acetic acid is added to the solution to keep a low pH. Haematoxylin has properties which closely resemble those of a basic dye. Basic dyes carry a positive charge which enables them to bind by electrostatic linkages to anionic groups within the tissue. When staining with haematoxylin, it is the mordant which carries the positive charge and gives the stain properties similar to a basic dye. The tissue-mordant-haematoxylin binding is not due simply to electrostatic charge and because of this, the dye does not dissociate easily from the tissue when placed in water and it is possible to counterstain with eosin (see below). Nuclei are stained blue with haematoxylin.
The sections were placed in the haematoxylin solution and left for 10 minutes. The slides were then rinsed in tap water until the water ran clear.

(3) Differentiation:- differentiation is the process of removing stain so that only the stain tightly bound to certain structures will remain. The preparations were dipped in acid alcohol for 5-10 s. The low pH of the alcohol causes the release of some of the dye from the stained tissue and leaves the nuclei stained.

(4) Rinsing:- the sections were left under running tap water until they turned blue. This is due to tap water having an alkaline pH and the haematein and potash alum react together under such conditions to produce the mordant-dye complex or lake.

(5) Staining with eosin:- the slides were immersed in eosin for 1 minute and then given a quick rinse with tap water to remove excess eosin. Eosin is an acid dye and as such it carries a negative charge. This dye binds electrostatically to the cationic groups of amino groups and counterstains the cytoplasm pink.

(6) Dehydration:- the sections were passed through graded alcohols to 100%; they were then passed through 2 changes of xylene. The preparations were passed briefly through the more aqueous alcohol solutions and left for 30 s in the changes of absolute alcohol.

(7) Mounting:- drops of DPX mountant (BDH, Poole, U.K.) were placed on the slides and coverslips placed over the sections, ensuring that there were no air bubbles trapped under the coverslips.

3.2.9 Cataracts.

Many of the STZ diabetic rats developed cataracts over the period of experimental study. Cataracts result from hyperosmolarity of the lens tissue. A photograph of a rat displaying such a cataract and by comparison, a non-diabetic control rat without cataracts is displayed in plate 3.7.

3.3 Results.

3.3.1 Changes in Body Weight.

Although there were sometimes large fluctuations in the weight of individual rats; when the weights were averaged for a group of animals that had been injected with STZ on the same day, the changes in average weight over 150 days followed the same pattern as groups of rats injected at different times. Figure 3.1 is a graph of the change in weight of two individual rats over 100 days, after the administration of STZ. Figure 3.2 shows the change in the average weight of a group of diabetic animals over 150 days, from the time of administration of STZ ($n = 27$). The animals whose weights are displayed in figure 3.2 were used in the experiments to find the peroneal cutaneous receptive field (see chapter 5). It can be seen from figure 3.2 that the average weight of the animals fell in the first two weeks after STZ administration. The weights then increased rapidly until approximately 50 days after administering the drug. After this time, there was only a very gradual increase in the average weight of the animals. The average weight of the diabetic animals whose weights are displayed in figure 3.2, was 475.32 ± 11.39 gwt at 149 days. The final average weight of an equivalent group of non-diabetic animals at 149 days was 524.60 ± 30.79 gwt.

Figure 3.1 A graph of the changes in body weight of two rats over 100 days after the administration of STZ

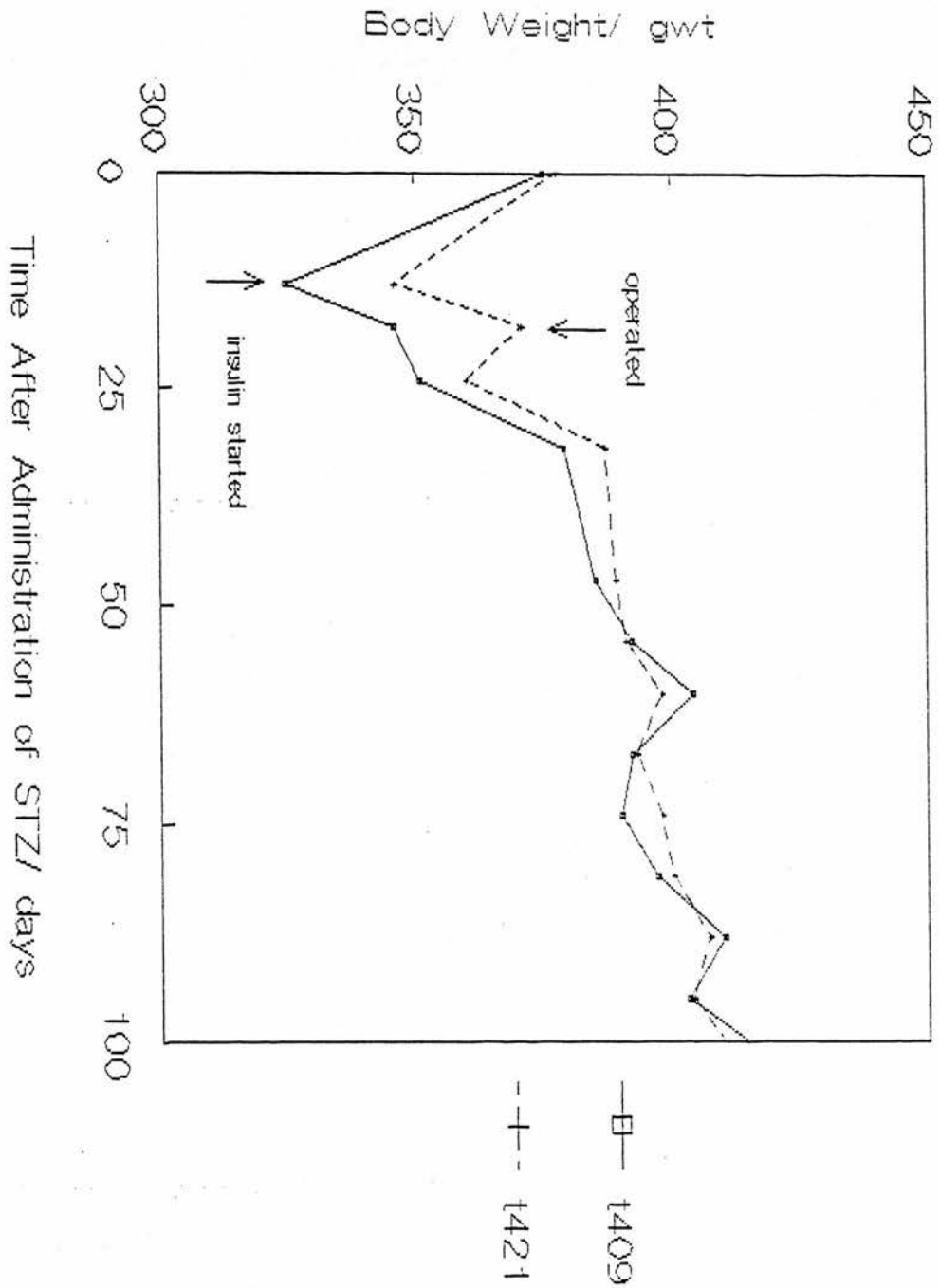
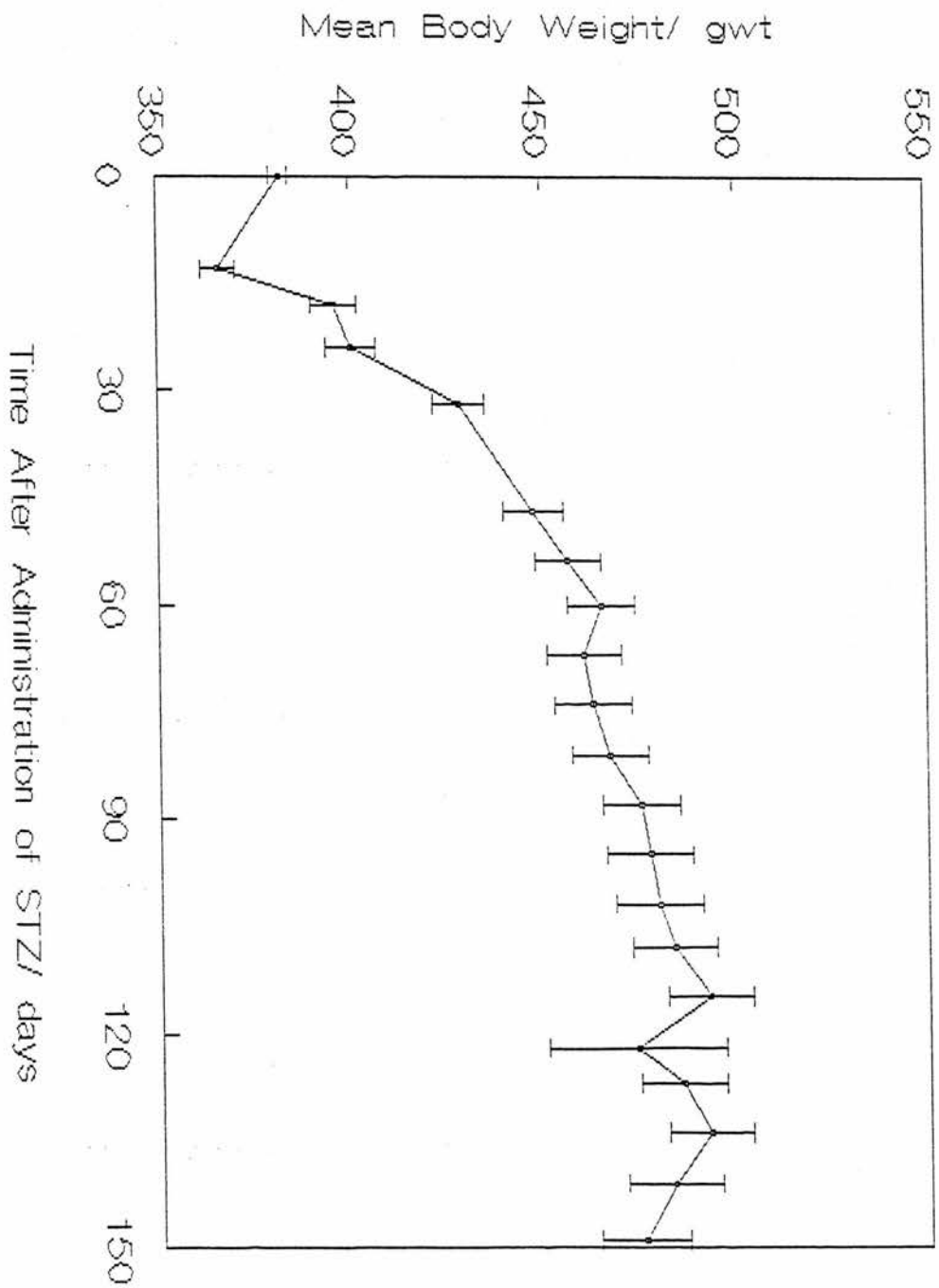


Figure 3.2 A graph of the change in the average weight (± SEM) of a group of STZ diabetic rats (group2; n = 27), over a period of 150 days after operating



3.3.2 Changes in Blood Glucose.

After induction of diabetes, the blood glucose levels were recorded every three weeks and when considered appropriate (see chapter 3.2.2). As described in chapter 3.2.2, insulin supplementation was given to those animals that lost more than 50 gwt in weight from their starting weights, and to those animals whose weight fell below 350 gwt. For these animals, the blood glucose levels were found to fluctuate considerably over the course of the experiments. This is because the levels of blood glucose inevitably fell immediately after insulin supplementation but rose to hyperglycaemic levels again some time prior to the next injection. The blood sugar levels recorded from a number of rats over 150 days, that had been injected with STZ on the same day, are displayed in table 3.1. These rats were used in the experiments of reflex tension that are described in chapter 7. The average values of blood sugar for the data presented in table 3.1 could not be calculated because the Reflolux S reflectance photometer measured blood glucose levels up to only 27.7 mM and, therefore, some glucose values were over the range that the meter could register. The values of mean blood glucose of the rats (presented in table 3.1) before administering STZ was 4.59±0.44 mM (n = 25). It can be seen from table 3.1 that the injection of STZ in citrate buffer induced a variable degree of hyperglycaemia in rats, however, only one animal had a blood glucose level below 14 mM (see chapter 3.4.2) and subsequently had to be rejected from further experimentation. Over time, the level of hyperglycaemia of each rat fluctuated considerably but was maintained at levels above normal. The mean blood glucose of a group of non-diabetic animals (used for the reflex tension experiments of chapter 7) before administering citrate buffer without STZ (see chapter 2.2) was 4.59±0.46 mM (n = 8). Two days after

administering the citrate buffer, the mean blood glucose level was 5.08±1.05 mM; 40 days later, the mean glucose level of the same 8 rats was 4.76±0.03 mM. It can be seen that the mean glucose levels were not affected by the administration of citrate buffer or over time.

Table 3.1 The blood sugar levels of rats (group 4), measured in mM,at different times after STZ administration (over: 30 mM)

TIME/ DAYS	0	2	8	14	40	62	86	108	129	150
RAT										
1	4.3	12.7	16.3	13.6	9.3	14.7	23.2	15.6	22	19.4
2	4.3	14.9	8.6	12.3	8.9	15.9	24.8	18.1	21.6	16.3
3	4.6	over	14.6	over	over	21.3	over	23.2	over	dead
4	4.8	over	17.3	13.6	14	14.8	over	6	26.6	dead
5	4.9	23.3	20.3	over	9.9	6.2	27.7	4.1	16	dead
6	4.8	15.9	19.8	over	8	15.3	over	3.7	15.7	4.5
7	4.5	21.8	over	27	24.7	13.2	12.2	19.8	16.6	13.5
8	6	21.5	26.4	13.8	22	12.6	13.2	24.3	10.8	dead
9	4	25.1	15.3	over	11.5	27.3	14.4	4.7	over	dead
10	4.8	18	over	over	9.3	11.2	-	20.1	16	dead
11	4.5	over	22.3	over	9.3	16.6	-	5.3	over	dead
12	4.3	over	15.9	over	10	over	-	7.6	over	dead
13	4.3	27.3	21.3	over	22.4	25.5	-	over	over	dead
14	4.2	over	14.8	over	23.5	over	-	14.7	over	dead
15	4.7	over	13.6	over	7.2	17.2	-	14.5	over	dead
16	4.4	over	15.7	over	18.3	10.2	-	18.5	11.3	dead
17	5.4	over	26.2	over	13.3	23.8	-	10.7	10.5	10.3
18	4.8	21	22	over	25.1	10.1	-	10.7	27.7	15.9
19	4.9	over	16.1	22.9	15.5	7.3	-	9.3	16.3	14.1
20	4.2	16.9	11.2	over	26.6	over	-	6.6	12.8	17.9
21	5.1	26.5	16.6	22.9	12.4	27.3	-	19.1	23.2	12.7
22	4.2	over	over	25.1	7.8	over	-	12.4	27.3	9.8
23	4.7	over	25.5	23.1	8.5	22.1	-	10.2	23.8	10.4
24	4.1	18.7	over	22.6	14.9	over	-	17.3	over	15.7
25	4.6	22.3	17.2	21.3	13.6	over	-	12.9	17.9	15.6

3.3.3 Changes in the Pancreas.

Non-diabetic rats.

The β cells of the islets of Langerhans of control pancreases stained densely for insulin. Large numbers of β cells were found in each islet although numbers varied between islets. The β cells were often found to be situated centrally within the islet and the non-β cells (α and δ) were situated at the periphery of the islet. The appearance of the islets was the same for animals whose pancreases had been removed 5 days after administration of citrate buffer as those animals whose pancreases had been removed 150 days after administration of citrate buffer. Plate 3.1 is a photograph of an islet of Langerhans from a rat that had been injected with citrate buffer 5 days before the removal of the pancreas (stained with haematoxylin and eosin). Plate 3.2 is a photograph of an islet of Langerhans from the pancreas of a non-diabetic rat that had been injected with citrate buffer 5 days previous to its removal (stained with monoclonal antibody for insulin).

Diabetic rats.

The sections of pancreas removed from the rats that had been administered with STZ 5 days earlier still contained pancreatic islets that stained positive for insulin. However, the numbers of islets appeared to be fewer and the numbers of β cells were certainly much fewer than those observed on the sections of pancreas removed from non-diabetic animals. Some islets were particularly small in size as compared to those of non-diabetic animals. Plate 3.3 is a photograph of an islet of Langerhans from a rat that had been injected with STZ 5 days before the removal of the pancreas (stained with haematoxylin and eosin). The blood glucose at the time of removing the pancreas was too high to be registered by the

Reflolux S reflectance photometer. Plate 3.4 is a photograph of an islet of Langerhans from a pancreas of a rat that had been injected with STZ 5 days previous to its removal (stained with monoclonal antibody for insulin). The blood glucose was 23.4 mM at the time of removing the pancreas.

150 days after administration of STZ, islets of Langerhans containing β cells were still found. In some cases there were numerous β cells which stained densely for insulin, however, islets appeared smaller and fewer in number than were found in non-diabetic animals of equivalent age. Plate 3.5 and plate 3.6 are photographs of endocrine pancreatic tissue stained with monoclonal antibodies for insulin. The former was from a diabetic animal whose pancreas had been removed 150 days after administration of STZ, blood glucose = 14.9 mM at the time of removing the pancreas; the latter was from a non-diabetic animal of equivalent age, blood glucose = 4.9 mM.

3.3.4 Cataracts.

Many of the rats that had been injected with STZ developed cataracts, sometimes in both eyes. The total numbers of rats that developed cataracts was not counted, however, none of the rats that were injected with citrate developed the condition. The number of animals that developed cataracts increased over the duration of the experiments. Plate 3.7 is a photograph of: (a) the eye of a non-diabetic rat without cataracts and (b) the eye of a diabetic rat with a cataract.

Plate 3.1 Photograph of an islet of Langerhans from a rat injected with citrate buffer 5 days before the removal of the pancreas (haematoxylin and eosin; X100)

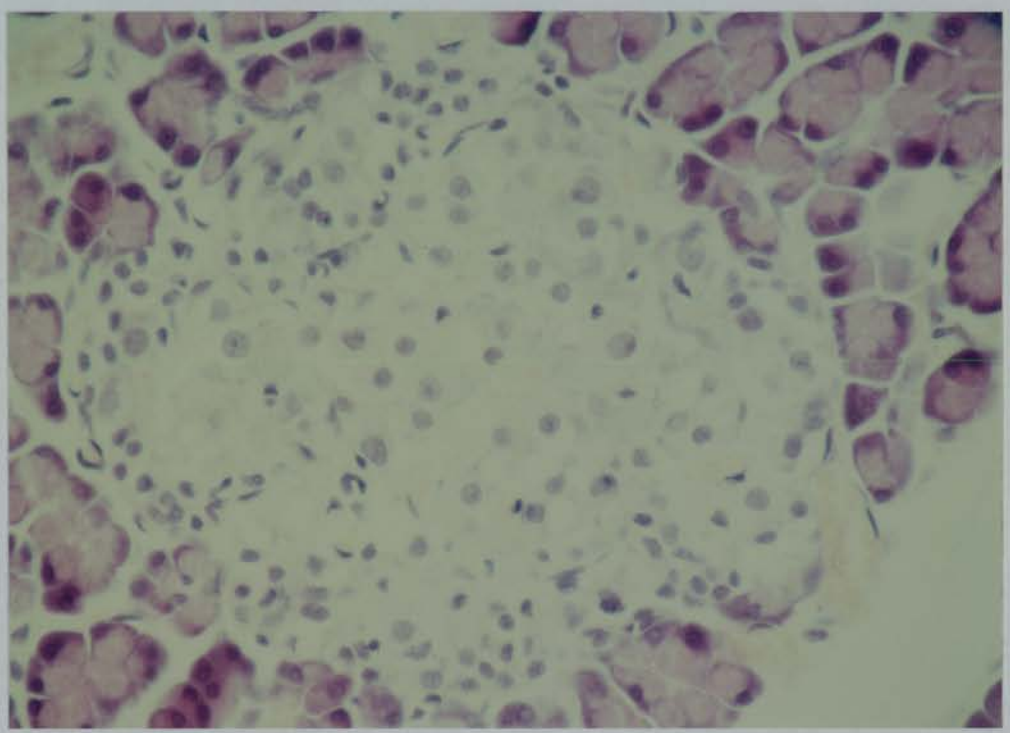


Plate 3.2 Photograph of an islet of Langerhans from a rat injected with citrate buffer 5 days before the removal of the pancreas (monoclonal antibody for insulin; X100)

Plate 3.3 Photograph of an islet of Langerhans from a rat injected with STZ 5 days before the removal of the pancreas (haematoxylin and eosin; X100)

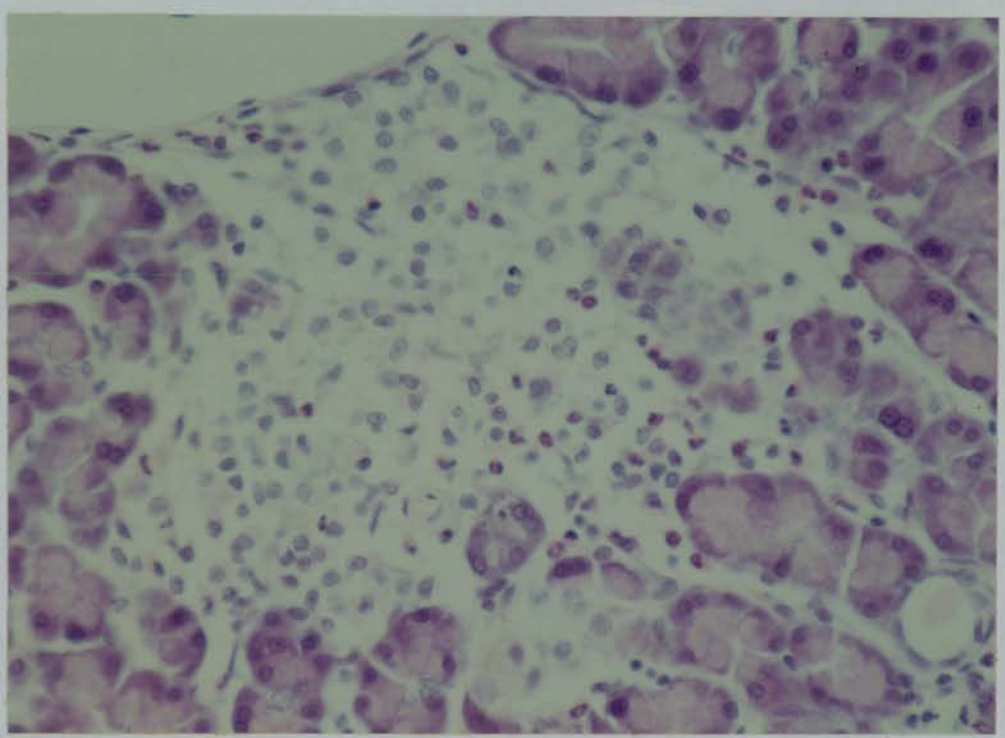


Plate 3.4 Photograph of an islet of Langerhans from a rat injected with STZ 5 days before the removal of the pancreas (monoclonal antibody for insulin; X100)

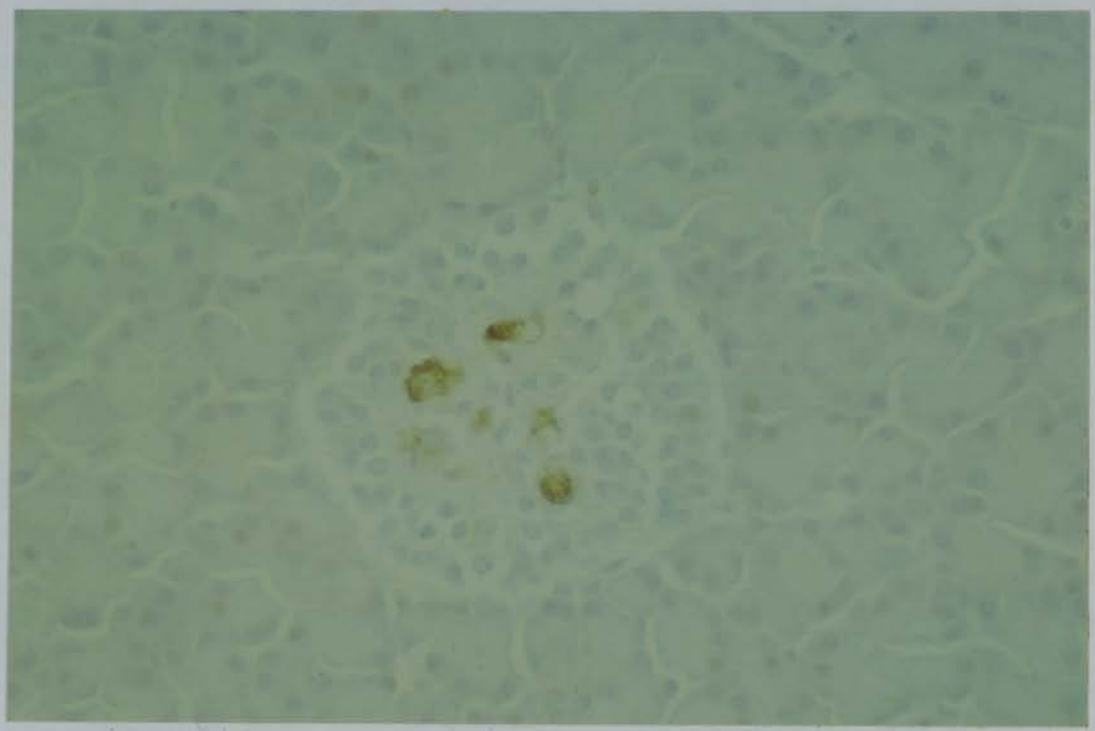


Plate 3.5 Photograph of endocrine pancreatic tissue from a rat injected with STZ approximately 150 days before the removal of the pancreas (monoclonal antibody for insulin; X40)

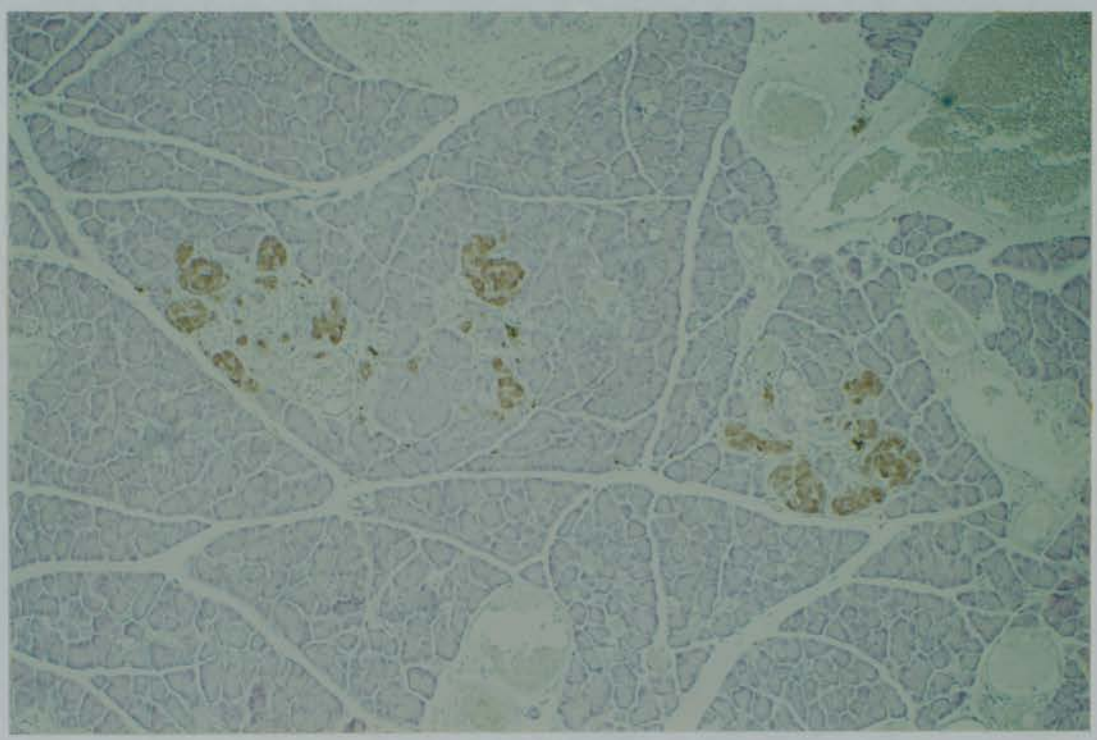


Plate 3.6 Photograph of endocrine pancreatic tissue from a rat injected with citrate buffer 150 days before the removal of the pancreas (monoclonal antibody for insulin; X40)

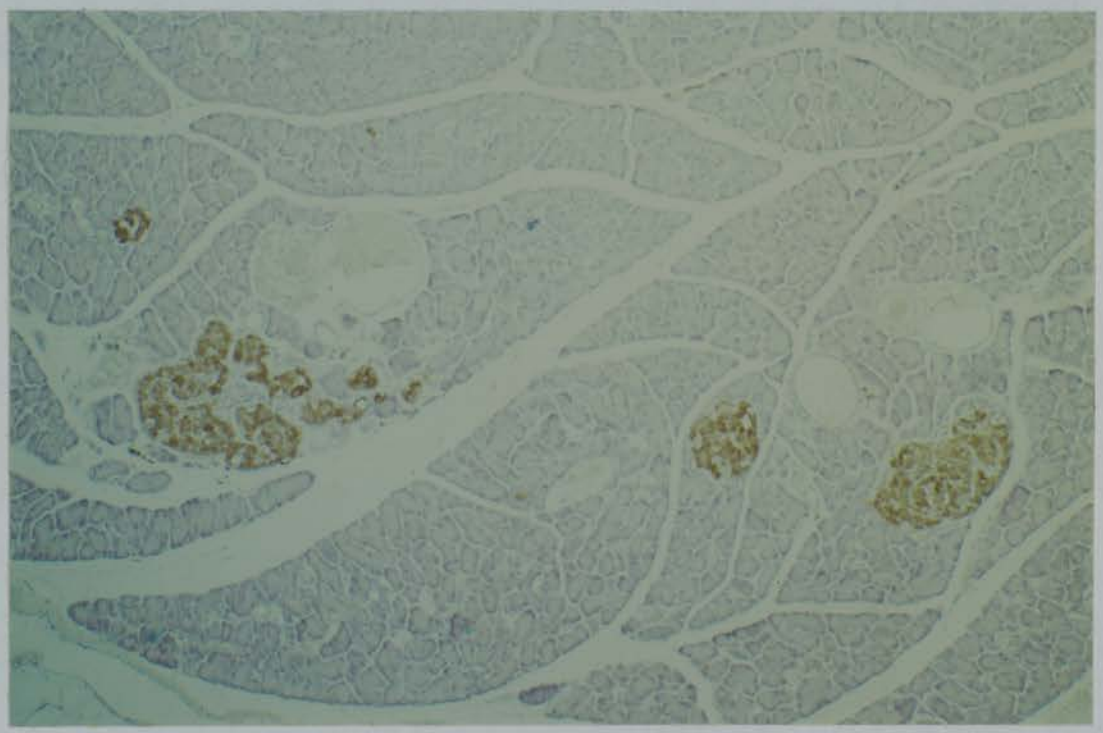
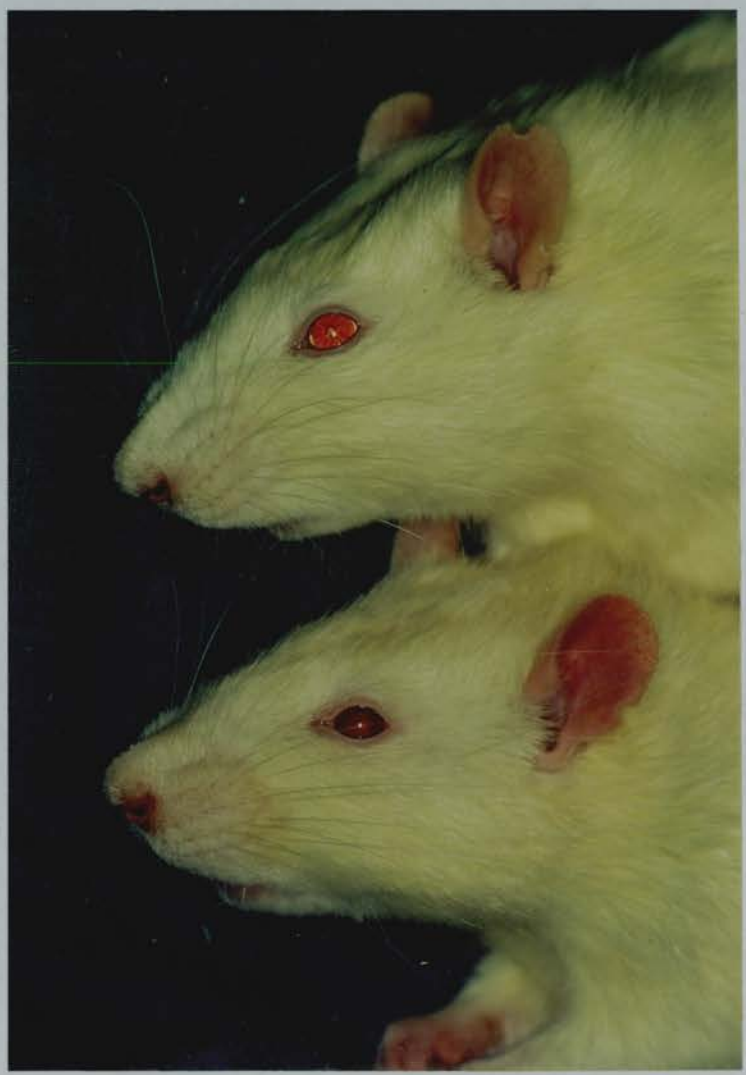


Plate 3.7 Photograph of (a) the eye of a non-diabetic rat without cataracts and (b) the eye of a diabetic rat with a cataract



3.4 Discussion.

3.4.1 Changes in Body Weight of the Animals.

The STZ diabetic animals in the experiments described in this thesis gained weight in the order of 100 gwt greater than their starting weight, over the course of the experiments. This is in contrast with the non-diabetic animals which gained weight in the order of 150 gwt over the same time period. Other researchers have reported much greater losses in weight in their STZ diabetic animals, however, their experiments were commonly of shorter duration and the long-term health of the animals was seemingly less of a concern. For example Jakobsen *et al.* (1981) reported losses of 50 ± 16 gwt in 1 week and 126 ± 34 gwt in 4 weeks after administration of STZ. However, McLean *et al.* (1987) showed much smaller losses of 15 gwt over 14 days. Ekström *et al.* (1989) observed changes in the weight of their STZ diabetic rats that were very similar to those observed in the experiments of this thesis. These authors found that the weight of the rats decreased over the first few days after inducing diabetes, after which time there was an increase in body weight until 20 days. In figure 3.1, the initial decrease in weight of 2 rats used in the work of this thesis after the administration of STZ, is clearly seen. Similar falls in weight were observed in all of the animals used in the work presented here.

Ekström *et al.* (1989) found that insulin treatment, started 20 days after the injection of STZ, caused a sharp increase in the gain in weight of their rats, whereas, no insulin treatment was followed by a progressive loss in weight until 28 days when the animals were killed. Unlike the insulin

treatment of Ekström *et al.* (1987), the supplementary doses of insulin that were given to the rats in the experiments presented here, were designed to prolong the lives of the animals and not to stabilize the blood glucose. Consequently, the gain in weight of the animals in the experiments presented here was not nearly as rapid nor as large as the gain in weight of the rats used by Ekström *et al.* (1987). Bisby (1979) also observed changes in the weight of his STZ diabetic rats that were similar to the changes observed in the experiments of this thesis. He found that there was an initial loss in weight followed by maintenance of a fairly constant weight.

3.4.2 Changes in Blood Glucose.

Ekström *et al.* (1989) chose a level 13 mM glucose, measured from blood removed from the tail of their STZ diabetic rats, as the cut-off for diabetes. Both Bisby (1980) and Jakobsen *et al.* (1981) used 300 mg dl^{-1} as the cut-off for their experiments on nerve regeneration in STZ diabetic rats (normal range is 90-150 mg dl^{-1}). 14 mM glucose, measured from blood removed from the tails of the rats (see chapter 3.2.3), was used as the cut-off for diabetes in the experiments in this thesis.

The average whole blood glucose in non-fasting rats is quoted to be around 5 mM (McLean *et al.*, 1987). The average blood glucose of the animals used in the experiments presented in this thesis was around 4.59 mM (see chapter 3.3.2). The average blood serum glucose of the non-diabetic rats of Ekström *et al.* (1989) was found to be 7.2±0.4 mM and that of their STZ diabetic rats was 28.8±2.3 mM. Similarly the mean plasma glucose of the control rats used by McLean *et al.* (1987) was 5.92±1.77 mM and that of the STZ diabetic rats was 26.03±13.2 mM. Although the mean blood glucose of the STZ diabetic rats used in the work of this thesis could not be calculated (owing to the limited range of the Reflolux S reflectance

photometer), it can be seen from table 3.1 that only 6 of the 25 rats had a blood glucose level less than 20 mM.

Similar blood glucose results were obtained in all of the rats used in the work presented in this thesis after administration of STZ. Although the blood glucose of the rats used in the present work fluctuated throughout the duration of the experiments, much of the variation was due to timing of testing the blood glucose after the injections of insulin. However, from figure 1 in Ekström *et al.* (1989), it can be seen that the blood glucose of the STZ diabetic rats of these authors fluctuated without insulin treatment. Other authors (Bisby, 1979; Jakobsen *et al.*, 1981; Longo *et al.*, 1986 and Triban *et al.*, 1989) only measured the blood glucose at the beginning and end of the experiments.

3.4.3 Changes in the Pancreas.

The distribution of β cells in the pancreas of the non-diabetic animals of the experiments presented here was similar to that described by Like and Orci (1971) in that the β cells were often found distributed centrally within an islet. Large areas of pancreatic tissue stained for insulin which indicated that insulin production was quite normal in the animals that had been injected with citrate buffer only. The staining of pancreatic tissue removed from non-diabetic animals provided good control material against which to compare the changes in the islets of Langerhans and numbers of β cells in pancreas removed from animals that had been injected with STZ.

Brosky and Logothetopoulos (1969) showed that there were no significant changes in the islets of Langerhans of mice 4-5 days after administration of STZ. After this time, they found results very similar to those observed in the rats in the experiments presented here. Brosky and Logothetopoulos (1969) observed that the numbers of islets were fewer and

those remaining were smaller containing fewer β cells than the islets of control pancreatic tissue. Furthermore, as in the results of the experiments presented in this thesis, the same authors found the occasional islet that contained larger numbers of β cells.

It was difficult to judge the distribution density of islets within the pancreatic tissue of the rats used here because only small samples were stained from pancreases removed from non-diabetic rats and from rats which had been injected with STZ. However, the small numbers of β cells seen in some islets of Langerhans (see plate 3.4) and the diminished size of many islets (see plate 3.3), as compared to control tissue, clearly demonstrates that STZ had an effect in destroying the normal histology of the pancreatic islets. These results combined with the high blood glucose levels that were recorded from the rats injected with STZ is evidence that the desired state of experimental diabetes had been induced in the rats used in the work of this thesis.

Brosky and Logothetopoulos (1969) demonstrated that mitotic activity of β cells of mouse pancreas increased during the first two weeks after STZ administration but by the end of two months, the proportion of cells undergoing mitosis was similar to that found in controls. They also demonstrated that guinea pigs did not recover from the diabetic state 8 months after the administration of STZ. The results of the work of this chapter corroborate the claims of Brosky and Logothetopoulos (1969) that the diabetic state induced by STZ is not reversed. Reduced numbers of β cells within islets of Langerhans from pancreas removed from rats 150 days after administration of STZ suggests that extensive regeneration of β cells had not occurred. In some islets, however, there was quite dense staining for insulin. This may be because granules containing insulin had not been released from β cells, owing to the fact that supplementary injections of

insulin had been given to the animals. In consequence, there was sufficient insulin present within the β cells for the binding of monoclonal antibody.

It is concluded that a state of hyperglycaemia had been induced in the rats used here and this was similar to diabetes mellitus in humans. The survival of the diabetic animals for the length of the experiments was ensured by giving some rats supplementary injections of insulin. The diabetic state induced by STZ is irreversible and was maintained in the rats for more than 150 days in a similar manner to very poorly controlled diabetes mellitus in humans. The similarity between STZ induced diabetes and type I diabetes mellitus has been discussed (see chapter 3.1.4) but in terms of hyperglycaemia, the state of diabetes induced in the rats of this thesis was very similar to both type I and type II diabetes mellitus in humans. Therefore, the STZ rats used in the work presented here provided a reliable model of poorly controlled human diabetes mellitus and were used in the experiments presented in the subsequent chapters of this thesis to compare recovery of function after nerve injury and repair.

CHAPTER 4

Physiological and Morphometric Assessment of Repaired Nerves

4.1 Introduction.

4.1.1 Aims.

The aims of this chapter are to describe the investigations of differences in the recovery of sciatic nerve conduction velocity and nerve morphometry after the different types of nerve injury and repair (described in chapter 2.5), and to compare the recovery, after 150 days, with similar repair groups of diabetic animals.

The recovery of conduction velocity depends on many different factors, and of greatest interest in the work presented here, is the nature of the injury. There are many reports on recovery of conduction velocity after nerve crush and nerve-to-nerve suture, however, the literature on recovery after grafting is somewhat scarce.

4.1.2 Changes in Nerve Conduction Velocity after Nerve Injury.

Erlanger and Schoepfle (1946) stated that after nerve injury and repair, full conduction velocity is never regained. The basis of the reduction in nerve conduction velocity lies, in part, with the structural changes that occur after nerve injury:

Berry, Grundfest and Hinsey (1944) demonstrated that the initial reduction in nerve conduction velocity, after nerve severance and suture, became less marked as the degree of nerve recovery became more advanced, and this occurred with a concomitant increase in the size of fibre diameter. Hursh (1939) demonstrated that the conduction velocity of mammalian fibres was directly proportional to fibre diameter, as did Berry,

Grundfest and Hinsey (1944). Cragg and Thomas (1961) also suggested that conduction velocity was most closely correlated with fibre diameter, whereas, Sanders and Whitteridge (1946) proposed a closer correlation between velocity and myelin thickness. However, Birren and Wall (1956) pointed out that the relationship between conduction velocity and fibre diameter is very hard to establish, particularly as measurements of morphological indices of regeneration vary with the type of preparation of the tissue; the outside diameter of fibres and myelin sheath thickness are both larger for osmic acid fixation than for freeze-dried tissue. In the case of unmyelinated axons, Hoffmeister, Janig and Lisney (1991) proposed a linear relationship between conduction velocity and axonal circumference.

In fact, conduction velocity is affected by both: (a) axon diameter and (b) myelin thickness. Action potentials are propagated discontinuously, in saltatory jumps, between one node of Ranvier and the next. Propagation of an impulse depends on a suprathreshold potential not 'fading' between nodes. A smaller depolarizing current at a node causes a delay in its reaching suprathreshold potential and hence, produces conduction slowing.

(a) Effect of axon diameter on conduction velocity.

As a current passes along a nerve fibre, potential is lost in overcoming the resistance of the axon. The resistance of an axon is inversely proportional to its transverse cross-sectional area. Large diameter axons have lower resistance than smaller axons and, therefore, the potential 'falls off' less over a given distance.

(b) Effect of myelin thickness on conduction velocity.

The insulation produced by the myelin sheath of nerve fibres is of high resistance and low capacitance. The capacitance of the myelin sheath is inversely proportional to its thickness. If a fibre loses myelin or is

demyelinated, a situation of low resistance and high capacitance is produced and the fibre is effectively short-circuited. Now, a smaller depolarizing current reaches the next node.

Apart from fibre and axon diameters, the internodal length is also important in governing the conduction velocity of nerve fibres. Tasaki (1953) proposed that according to the theory of saltatory conduction, it would be expected that conduction velocity would be equal to internodal length divided by the transmission time i.e. the delay between the times at which adjacent nodes reach action potential threshold.

Although ultrastructural changes in nerve fibres could affect their electrical properties, this possible difference between control and regenerated nerve is not addressed in the work presented in this thesis.

4.1.3 Recovery of Nerve Conduction Velocity after Nerve Injury.

Cragg and Thomas (1964) investigated the change in conduction velocity distal to a nerve crush injury, in the peroneal nerves of rabbits. They showed a return of conduction velocity to approximately 75% of control values, 12-16 months after the injury was incurred. This is in contrast to the findings of Sanders and Whitteridge (1946) who found that after a similar injury and recovery time, the conduction velocity of their rabbits had returned to values indistinguishable from controls. The site at which electrophysiological measurements are made is of the utmost importance. In a different paper, Cragg and Thomas (1961) reported the changes in conduction velocity proximal to nerve crush and nerve-to-nerve suture injuries of the rabbit peroneal nerve, over 446 days. Their findings showed that after both types of injury, conduction velocity decreased to 80% of normal values around 50-100 days and recovered normal conduction

velocities by 200 days. Myles and Glasby (1991) investigated the recovery of sciatic nerve conduction velocity after nerve-to-nerve suture, three strand cable isograft and muscle grafts, in rats. These authors also failed to demonstrate a return to control values of conduction velocity, even after 300 days.

It is known that nerve crush injury is followed by a decrease in axon and fibre diameter (Gutmann & Sanders, 1943). Similar to the findings of Cragg & Thomas (1964), in relation to the recovery of conduction velocity (above), Gutmann and Sanders (1943) found that there was a gradual improvement in fibre size with recovery time, although fibre size never returned to control values. This suggests that the structural changes may contribute to the decrease in conduction velocity.

4.1.4 Recovery of Nerve Morphology after Nerve Injury.

Similar to the recovery of conduction velocity after nerve injury, accounts of recovery of fibre diameter after nerve crush and nerve-to-nerve suture are more numerous than those of recovery after nerve graft.

Gutmann and Sanders (1943) produced an extensive account of recovery of fibre number and diameter after nerve crush, nerve-to-nerve suture and nerve graft in rabbits. They found that 150-200 days after nerve crush, fibre numbers distal to the injury were approximately equal to those found proximal to the injury. After nerve-to-nerve suture and nerve grafting, numbers of nerve fibres never reached control values. However, the total fibre count is not a particularly useful measure as an indication of the degree of recovery of nerves after injury. Even in control nerves, it has been shown that differences in numbers of fibres in the sciatic nerve of rats can vary between animals by 10% (Weiss & Cambell, 1944). During the regeneration

process numerous sprouts are produced by each fibre (see chapter 1.1) and can lead to misleading results of the nerve count.

Gutmann and Sanders (1943) also discovered that after nerve crush, nerve-to-nerve suture and nerve graft, there is a reduction in the diameter of nerve fibres proximal to the injury. After nerve crush, the reduction in fibre diameter is progressive until approximately 130 days, after which time there is a gradual increase to normal values. Similar reductions in fibre diameter were observed by Cragg and Thomas (1961), proximal to nerve crush and nerve-to-nerve suture injuries, in the peroneal nerve of rabbits. However, Cragg and Thomas (1961) found a return of fibre diameter size to near normal values after nerve-to-nerve-suture; no secondary increase in fibre diameter was found by Gutmann and Sanders (1943) after nerve-to-nerve suture or nerve graft. The reduction in fibre diameter and the subsequent return to normal values proximal to a crush injury, could explain the reduction and return of conduction velocity that was noted by Sanders and Whitteridge (1946) (see above).

Distal to a crush injury, Gutmann and Sanders (1943) demonstrated that fibre diameter gradually increased until normal sizes were attained by 250-300 days. After nerve-to-nerve suture and nerve graft, fibre sizes remained smaller than those proximal to the injury, even 1 year later. They found that the distribution of fibre size in control animals had 2 peaks; after nerve crush the peak corresponding to the larger fibres did not reappear until 200 days and after nerve-to-nerve suture and nerve graft, not at all. Similarly to the findings of Gutman and Sanders (1943), Berry, Grundfest and Hinsey (1944) found that fibre diameter, measured up to 466 days after peroneal, tibial and saphenous nerve severance and nerve-to-nerve suture in cats, never regained normal values.

Myles and Glasby (1991) demonstrated that after nerve-to-nerve suture, three strand cable isograft and muscle graft of sciatic nerves in rats, control values of axon and fibre diameter were not attained by 300 days.

4.1.5 Nerve Conduction Velocity in Diabetes.

A decrease in nerve conduction velocity is one of the most important and earliest recognized functional changes that occurs in diabetic peripheral nerve. In 1964 Eliasson induced experimental diabetes in rats by administering alloxan; he demonstrated a reduction of conduction velocity in the sciatic nerves of these animals similar to that produced after pancreatectomy. The reduction in conduction velocity is reported to be up to 25% to 30% of the values recorded from non-diabetic animals (Eliasson, 1964; Greene, De Jesus,and Winegrad, 1975; Jakobsen, 1979), although Thomas, Jefferys, Sharma and Bajada (1981) demonstrated a reduction of only 2.2 ms^{-1} in mature rats that had been injected with STZ.

It is generally accepted that in the early stages of IDDM diabetes, the decrease in conduction velocity is reversible with insulin treatment (Gregerson, 1967). There are also reports of the reversal of the decrease in conduction velocity in experimental diabetes, with insulin treatment (Jakobsen, 1979; Ward, Bowes, Fisher, Jessop and Boher, 1971) and myoinositol supplementation (Greene *et al.*, 1975). Although others have failed to produce a reversal of the decrease in nerve conduction velocity with both insulin (Eliasson, 1964) and myoinositol supplementation (Jefferys, Palmano, Sharma and Thomas, 1978). As the disease progresses, the decrease in conduction velocity can no longer be reversed (Sima, Yagihashi and Greene, 1990). This irreversibility of conduction velocity is also true of human diabetics once neuropathy has been established (Pietri, Ehle and Raskin, 1980).

4.1.6 Pathogenesis of Nerve Conduction Decrease in Diabetes.

The different theories which may explain the neuropathic changes that occur in peripheral nerve of diabetics have been discussed in chapter 1.7. It is probable, however, that the decrease in conduction velocity results from a combination of factors. In this section, the various proposed causative factors of the nerve conduction decrease will be discussed; however, the aim of this chapter is primarily to investigate 'by how much' conduction velocity returns after nerve repair in diabetic rats as compared to non-diabetics, as a means of evaluating the degree of recovery after nerve injury and repair.

Thomas (1971) said that the basis of the decrease in conduction velocity seen in diabetic neuropathy is very likely to be due to the extensive demyelination that is characteristic of the disease and the associated axonopathy. A selective loss of large myelinated fibres and a change in myelination could lead to reduced conduction velocity. Segmental demyelination is rarely seen without a degree of axonal degeneration and *vice versa* (Thomas & Lascelles, 1966). This suggests that there is a relationship between axons and Schwann cells such that either a decrease in axon diameter leads to a breakdown in myelin (Young, 1944) or Schwann cells provide some kind of metabolic support for axons. However, the pathological changes that are apparent in human diabetic neuropathy are not as obvious, if at all, in experimental diabetes, yet, conduction velocity changes do occur in animal models. Thomas himself, in association with Sharma, failed to find any signs of segmental demyelination or a decrease in fibre diameter in STZ diabetic rats (Sharma & Thomas, 1974). In addition, the reduction in conduction velocity is apparent very soon after diabetes has been induced in experimental models. Greene *et al.* (1975) noted a

decrease in motor nerve conduction velocity 14 days after STZ injection in rats, too soon for morphological damage of the nerve to have occurred. It is unlikely that the morphological changes are solely responsible for the huge decreases in conduction velocity that are observed in diabetes.

It is probable that the morphological changes of diabetic nerve are, in part, responsible for the irreversibility of the decrease in nerve conduction velocity seen in longer term diabetes. Sima *et al.* (1980) demonstrated that although sural nerve fibre diameters were normal after vigorous treatment of spontaneously diabetic BB Wistar rats with insulin, conduction velocity values were only partially returned to normal. A recent explanation for the irreversibility of the decrease in conduction velocity is the migration of paranodal axo-glial junctional complexes (Sima, Lattimer, Yagihashi and Greene, 1985). Sima et al. (1985) demonstrated in spontaneously diabetic BB Wistar rats treated vigorously with insulin, that although normalization of sural nerve fibre calibre, myoinositol and Na^+/K^+ ATP-ase levels were obtained, conduction velocity values were only partially restored. Notably, they found a disappearance of paranodal axo-glial junctional complexes, which did not return with insulin treatment. The function of the junctional complexes is thought to be to limit the migration of Na^+ channels away from nodes of Ranvier. If such migration of paranodal complexes occurs, this would account for a decrease in nodal Na^+ permeability which could explain the permanent reduction in conduction velocity.

The reversibility of decreased conduction velocity in acute experimental diabetes may have a biochemical basis. As discussed in chapter 1.7 (metabolic hypothesis), hyperglycaemia leads to an increase in intracellular glucose, sorbitol and fructose. Associated with this increase in polyol metabolism is a decrease in the levels of tissue myoinositol (MI). It is thought by some that the decrease in MI causes a change in Na^+/K^+

ATP-ase activity (Greene *et al.*, 1988). The decrease in Na^+/K^+ ATP-ase activity then affects the levels of axonal Na^+, which, in turn, affects the function of voltage-gated Na^+ channels. Consequently, subthreshold membrane potentials are produced causing selective conduction block of large myelinated fibres and a slowed conduction velocity. Some authors (Jeffereys *et al.*, 1978) have failed to substantiate the claims of Greene *et al.* (1975) that MI supplementation improves conduction velocity.

The proposed biochemical mechanism behind the effect of hyperglycaemia on the alteration in Na^+/K^+ ATP-ase activity, has been described by Winegrad and Simmons (1990). These authors proposed that hyperglycaemia inhibits active MI transport. But tissue MI is maintained by other mechanisms including: endogenous MI synthesis and recovery from MI-containing phospholipids (by hydrolysis). MI is a component of phosphatidylinositol (PI). Importantly, it seems that the cellular PI pool/turnover is dependent on this active transport of myoinositol and, although total tissue MI may be normal in the hyperglycaemic state, the inhibition of active MI transport affects the PI pool. PI itself is involved in the regulation of a distinct fraction of Na^+/K^+ ATP-ase activity. Hence, hyperglycaemia causes a decrease in Na^+/K^+ ATP-ase activity by inhibiting PI turnover, even when total tissue MI is normal.

Those in favour of the vascular theory as the cause of neuropathological changes have demonstrated that the decrease in nerve conduction velocity observed in diabetic animals, can be reproduced in non-diabetic animals in hypoxic conditions (Low *et al.*, 1986). Also, the failure to find a reduction in total nerve MI has led some researchers to dismiss the role of MI in causing a reduction of nerve conduction velocity (Dyck *et al.*, 1988), although total tissue MI may not be important, as described above.

4.1.7 Changes in Nerve Morphology in Diabetes.

It has already been mentioned above that the morphological changes seen in human diabetic neuropathy are not as apparent, if at all, in experimental neuropathy of STZ diabetic rats. Sharma and Thomas (1974) did not find any conspicuous morphological changes in their STZ diabetic rats, nor did Mendell, Sahenk, Warmolts and Marshall (1980), in their spontaneously diabetic BB Wistar rats although the latter authors found a reduction in nerve conduction velocity. This is contrast with Powell, Knox, Lee, Charters, Orloff, Garrett and Lampert (1977) who found demyelination and nerve fibre loss in the sciatic nerve of their alloxan diabetic rats. The differences in the findings of different groups of researchers might be due to the differences in the duration of diabetes of their animals. Alternatively, the changes reported by Powell, Ward, Garrett, Orloff and Lampert (1979) could be due to the ageing process. Thomas, King and Sharma (1980) found similar changes in ageing rats but the presence of intraaxonal glycogenosomes that were observed by Powell *et al.* (1979) are more commonly found in diabetic rats and not ageing rats.

Morphological changes have also been reported by Jakobsen (1976a) as soon as 4 weeks after STZ administration to rats. He noted a reduction in total fibre cross-sectional area, though he found smaller fibres were more greatly affected than large ones and axons were more greatly affected than myelin. He also found a decreased axon/myelin ratio and proposed that this may explain the decreased nerve conduction velocities observed in diabetic rats. He suggested that the observed 14% reduction in fibre area would correspond to a 7% reduction in fibre diameter. In a second paper Jakobsen (1976b) claimed to find a reduction in nerve fibre caliber in short-term experimental diabetes, though no evidence of segmental demyelination or

remyelination was found. He also observed a 27% widening of the nodes of Ranvier and paranodal swelling. Wattig, Warzok, Tglinicki and Hufnagl (1991) also found a decrease in cross-sectional area of myelinated fibres in the sural nerve of STZ diabetic rats, 5 to 6 weeks after induction of diabetes. However, their view differs from that of Jakobsen because they demonstrated a reduction in myelin cross section but found no change in axonal area. They proposed that the primary lesion in diabetic nerve is the Schwann cell.

Sharma, Thomas and De Molina (1977) failed to find any change in maximum or average myelinated fibre diameter 5 weeks after induction of diabetes with STZ. They proposed that the observed decrease in conduction velocity probably had a metabolic basis rather than a structural one. They also suggested that any reduction in fibre diameter might actually be because of poor health and a failure in the growth of diabetic animals. In 1979 Jakobsen confirmed his findings of reduced fibre diameters in STZ diabetic rats and also noticed a decrease in the cross-sectional axon area to myelin area relationship in diabetic rats as compared to their age-matched controls. This could be explained either by axonal shrinkage or by a failure of axons to mature during the experimental period. Sharma *et al.* (1977) did not find any alteration in the relationship between myelin sheath thickness and axon circumference, using a method involving the counting of myelin lamellae. In 1985, however, Dockery and Sharma did find reductions in fibre area and myelin area in STZ diabetic rats at 4 and 6 months duration of diabetes, and significant reduction in axon area at 6 months (although no differences were found at 2 and 4 months). Importantly, fibre area, axon area and myelin area did not differ from onset controls which led the authors to believe that the differences were, indeed, due to a failure of maturation of the diabetic nerve.

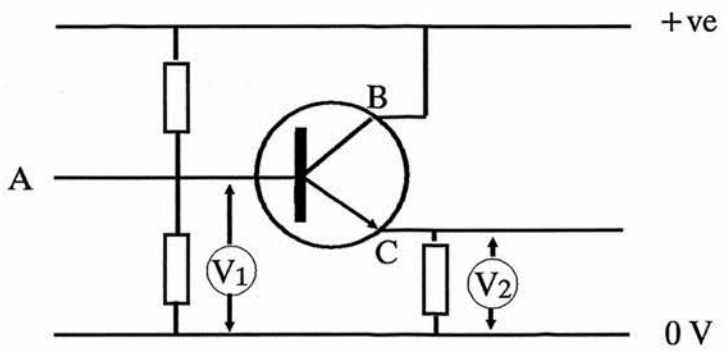
4.2 Materials and Methods.

4.2.1 Electrophysiological Equipment.

When recording small electrical signals from biological preparations, it is necessary to amplify those signals in order to improve their perception. Different types of amplifiers are used in different situations. The factor which governs the choice of amplifier for recording from peripheral nerve is that the nerve is a high impedance system. For nerve conduction this means dealing with large potential differences and relatively small currents. A small accumulation of current, as with static electricity, will produce large extraneous potentials which may interfere with those intended for recording. AC amplifiers are used in this study for the reason set out below:

Figure 4.1 shows a simple DC amplifier. In such a system a small current passing through the transistor from B to C will decrease its impedance. When a recorded potential produces a current at A, a larger current will flow through the transistor and the potential at V_2 will be greater than the recorded potential V_1. Hence amplification of the signal is produced. Incoming signals from biological preparations have an alternating current (AC) and a direct current (DC) component. The DC input can be affected by small artefactual currents, produced by static electricity for example; however, the AC component remains unaffected. Changes in the DC level caused by such noise is amplified by a DC amplifier.

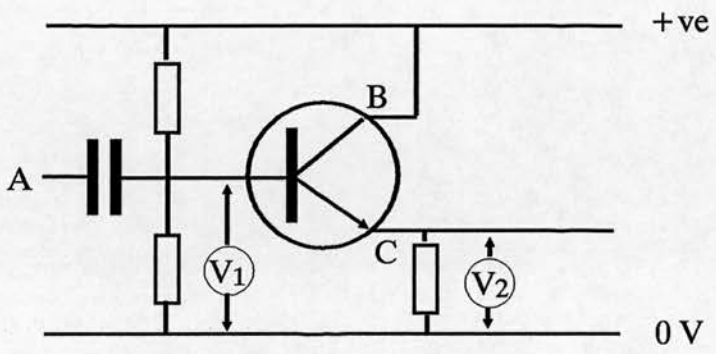
Figure 4.1 A simple DC amplifier



An AC amplifier as shown in figure 4.2 overcomes the problem of undesirable amplification of small artefactual signals. Amplification occurs in the same way as in a DC amplification system but the circuit of an AC amplifier contains a capacitor. By its very nature, the capacitor will store and release the AC component of the incoming signal; however, artefactually induced changes in the DC component of the incoming signal will be stored and not released by the capacitor. Therefore, only the alternating current will be released from the capacitor to be amplified and reflect the changes in potential that are occurring in the nerve as a result of stimulation.

The difference between DC and AC amplifiers, as a result of the insertion of the capacitor, is that the currents at V_2 will be 90^o ahead in a cycle.

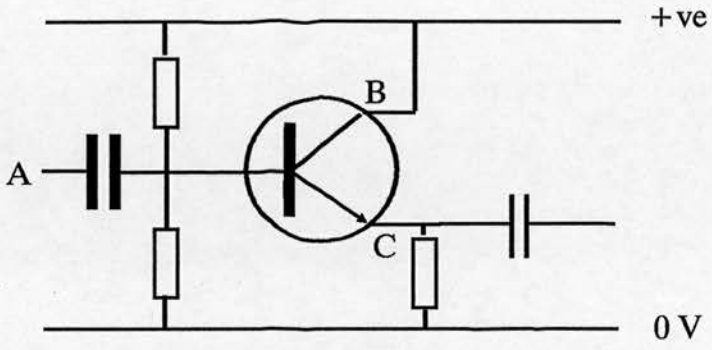
Figure 4.2 A simple AC amplifier



Filtering is of great importance so that only noise is filtered out and not the signal intended for recording. The signal can be filtered before amplificaton but in the present work filters were used after amplification. This is because the resistive components of the filters themselves introduce noise. If filters are incorporated into the circuit before amplification, the noise aswell as the signal is amplified. Filters put into the circuit after amplification will filter out the undesirable amplified noise from external sources and introduce relatively small amounts of noise themselves. Two filters: a high-pass and a low-pass filter, were used.

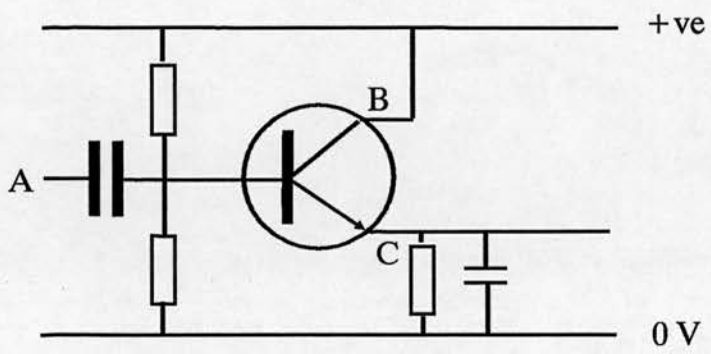
Filtering is performed by means of capacitors inserted into the circuit. The impedance of a capacitor increases with decreasing frequency of current passing through it. Also at a given frequency, the impedance is greater for a small capacitor as compared with a larger capacitor. If a small capacitor is placed in series as in figure 4.3, low frequencies will not 'get through' because of the large impedance. However, high frequencies will pass through the filter. This situation produces a simple high-pass filter.

Figure 4.3 Diagram to show the insertion of a capacitor to produce a high-pass filter



In figure 4.4, the small capacitor is placed in parallel. With this arrangement, at high frequencies, the impedance of the capacitor is low and a large fraction of the signal will pass through it; the signal to the outflow will, therefore, be small. At low frequencies, the impedance of the capacitor is increased, a smaller fraction of the incoming signal will pass through it and the signal to the outflow will be nearer to maximum. In this way higher frequencies are cut off and the circuit is a simple low-pass filter. High frequencies have a rapid rising phase and in the work presented here, where the waveforms are not sine waves, it is this rising phase which is of importance when it comes to filtering. The compound action potentials used in the measurement of conduction velocities have rapid rising phases so that in order not to filter out that part of the signal which is of interest, it is necessary to have the filters as wide open as possible. This is particularly so for the low pass filter.

Figure 4.4 Diagram to show the insertion of a capacitor to produce a low-pass filter



4.2.2 Preparation of Experimental Animals.

After the rats had been operated on as described in chapter 2.6, they were left for 150 days to allow nerve regeneration to take place. After this time, each rat was anaesthetized and the sciatic nerve on both sides was exposed (see sections 2.3 and 2.4). Each animal was kept warm on an electrically heated blanket and a digital temperature probe was placed in its rectum to monitor core temperature. The body temperature of the rat was kept as close to 37°C as possible by adjusting the heat produced by the blanket. 10 ml of 0.9% saline at 37°C was injected subcutaneously at the scruff of the neck to replace, by slow absorption, any fluid lost from the operation site. The exposed sciatic nerves were freed completely from the surrounding tissue-bed using microsurgical instruments and vision was aided by the use of an operating microscope (Weck Fibermatic 0902A1, Long Island City, New York). Freeing the nerve on the operated sides was more difficult because of scar tissue remaining from the previous operation.

This was found to be a particular problem in the muscle grafted animals. Each animal was placed, still on the heated blanket, in an earthed Faraday cage in order to reduce electrical interference in the subsequent recordings. The power to the blanket was turned off during recording because this was a significant source of electrical noise.

4.2.3 Stimulation of the Sciatic Nerve.

A bipolar, low impedance, palladium wire stimulating electrode was placed under the sciatic nerve at the point where the nerve emerges from the sciatic notch. The cathode of the electrode was always placed distal to the anode. A similar bipolar electrode was positioned under the sciatic nerve at a distance of about 2 cm distal from the stimulating electrode; the cathode here was placed in the anatomically 'proximal' position relative to the anode. Low impedance electrodes were used because the nerve is a high impedance system. Small currents passing through such a system will not be detected by electrodes of high impedance as charge will tend to 'take the pathway of least resistance'.

A silver wire ground electrode was placed in the muscle underlying the sciatic nerve. The table and Faraday cage were insulated from the floor; ultimately these were connected to ground by way of 'mains earth'.

The stimulating electrode was connected to a signal generator (Dagan 9200 Omni Pulse stimulator, Dagan Corporation, Minneapolis, U.S.A.) and a battery-powered Dagan 9250 stimulus isolator unit (Dagan Corporation, Minneapolis, U.S.A.). The 9200 Omni Pulse stimulator was programmed to produce an output TTL pulse every second, with a delay of 2 ms and duration of 50 μs. This was used to drive the stimulus isolator at which it was possible to control the amplitude of its square wave analogue output between 10 V and 100 V. The current output of the isolator, in this mode,

was variable. The stimulus isolator was connected to the stimulating electrode. A square wave supramaximal stimulus of between 10 and 20 V was used to stimulate the nerve.

The stimulus isolator contains several optoisolators. The optoisolators prevent the completion of an electrical circuit through the nerve preparation, thereby, preventing the introduction of artefactual pulses in the recording electrode. The optoisolators also allow impedance matching so that the stimulator can be used to produce a large voltage and small current, and *vice versa*.

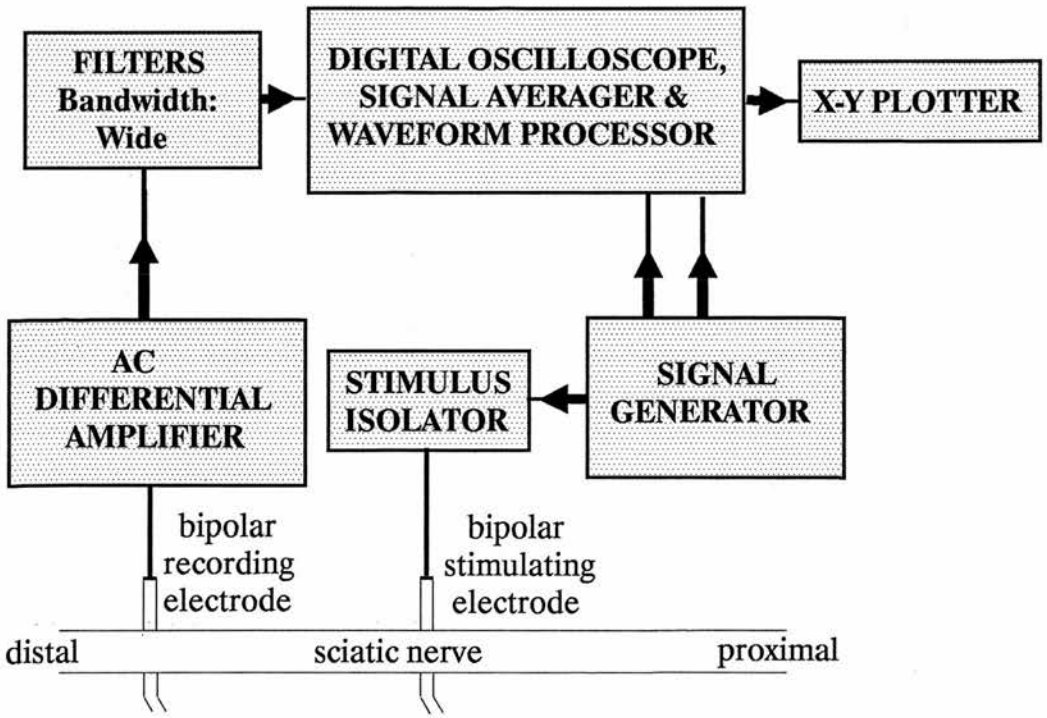
The Omni Pulse stimulator was used to trigger a Gould 4070 digital storage oscilloscope and Gould 740 waveform processor (Gould Electronics Ltd, Ilford, U.K.), and also to deliver a transistor logic timing pulse, synchronously with the stimulus, which was displayed on the oscilloscope. This trace was used as a square-wave stimulus marker, the upstroke of which provided a reference for timing the recorded action potentials. The delay was used simply to allow some baseline trace to be displayed on the oscilloscope betweeon the trigger and the stimulus marker.

4.2.4 Recording of Extracellular Action Potentials.

Figure 4.5 is a diagram of the electrophysiological stimulating and recording apparatus. The recording electrode was connected to the input of a Neurolog NL104A differential AC amplifier and NL125 filter (Digitimer Ltd, Welwyn Garden City, U.K.). The filters were kept as wide open as possible for reasons explained above. The output of the filter was connected to channel 1 of the y-inputs of the oscilloscope. The action potentials were displayed on the oscilloscope and the signal averaging facility of the waveform processor was used to average 8 traces. No more than 8 traces

were averaged because the nerve tires from overstimulation owing to the depletion of ATP stores. Averaging the action potentials decreased the possibility of recording an isolated anomalous event and also increased the signal-to-noise ratio.

Figure 4.5 Diagram to show the electrophysiological stimulating and recording apparatus



4.2.5 Measurement of Nerve Conduction Velocity.

The oscilloscope can be used to measure time intervals and also the amplitude of recorded signals by measuring the distance between datum lines and cursors. The time from the upstroke of the stimulus marker to the various positive peaks of the averaged compound action potential was measured. A stimulus artefact was always present and occurred at a time shorter than the first peak. However, if the timebase of the oscilloscope is too long it is very easy for the first peak to be hidden. If the time to the second peak is measured, much longer conduction velocities will be calculated. Therefore, in the experiments presented here, a short timebase was used. The stimulus artefact occurs as a result of rapid conduction of current from one electrode to the other via surrounding connective tissue and electrolytes. A typical trace of a recorded action potential is shown in figure 4.6. The distance between the cathodes of the stimulating and recording electrodes was measured as accurately as possible using calipers. From the equation:

$$v = s/t$$

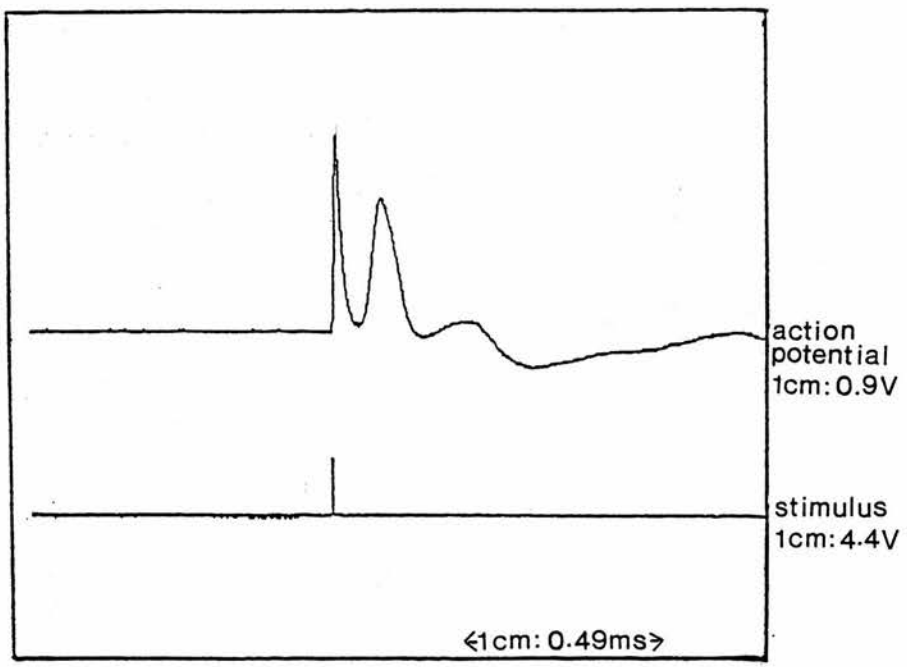
where: v = velocity/ ms^{-1}

s = distance between cathodes/ mm

t = time from upstroke of stimulus marker to first peak of action potential/ ms

the velocity of conduction of the fastest fibres was calculated.

Figure 4.6 A trace of an action potential recorded from a rat sciatic nerve from the non-diabetic control group



4.2.6 Measurement of Excitability of Nerve.

Chronaxie (see below) may be used as a measurement of nerve excitability (Myles & Glasby, 1991). Chronaxie values for the sciatic nerve were found from strength-duration curves obtained by stimulating at the nerve at various amplitudes for different lengths of time. For a given duration of stimulus/ ms, there is a value of strength of stimulus/ V, at which there is an all-or-nothing response.

The relationship between the voltage required to excite a nerve and the duration of the stimulus is approximately exponential. For long durations of stimulus, the potential required to elicit an all-or-nothing electrical response in the nerve is called rheobase, or V_o. This threshold value of voltage is susceptible to variation as it depends on the preparation as well as on the nerve itself (Myles & Glasby, 1991). A much more reliable measurement as an indicator of nerve excitability is chronaxie. This is the time required for a stimulus of twice rheobasic voltage to be effective for a constant response.

The aparatus was set up as in figure 4.5 as for measuring conduction velocity. The Dagan Omni Pulse stimulator was programmed to produce incrementing pulses at a frequency of 1 Hz. The duration of the stimulus was set, ranging between 0.001 ms and 5 ms. For each duration of the stimulus the amplitude of the pulses was incremented sequentially until an all-or-nothing action potential was first observed on the oscilloscope screen. At this point the sequence of pulses was paused and the stimulus amplitude required to produce an action potential at each given duration was noted. The second velocity peak of the action potential was used because the first peak was inconsistent in size and was easily confused with the stimulus artefact (see above). A graph was plotted of stimulus amplitude/ V against

duration/ ms. The voltage below which no action potential can be elicited, however long the stimulus, is called the rheobasic voltage.

Rheobase is used in the standardization of strength-duration curves so that one curve may be compared to another. This standardization is achieved by plotting $\log_{10}$(stimulus duration/ ms) against (strength of the stimulus/ V)/ (rheobase/ V) i.e. V/V_o. For long durations $V = V_o$, therefore, the curve always falls exponentially to $y = 1$. The values of duration are expressed logarithmically so that a standard scale of -2 to +2, (units = $\log_{10}$ ms), is obtained and the curves will be contained within the same abscissa. Families of curves from animals in different groups can then be compared. An example of such a curve is shown in figure 4.7. In this work, the graphs were plotted using Lotus Symphony software, Release 3 (Lotus Development corporation, U.S.A.) on a PC (Zenith Data Systems Corporation, Michigan, U.S.A.). The graph was then imported into Slidewrite software, Version 3 (Advanced Graphics Software Inc., California, U.S.A.) and an exponential curve was fitted to the points using this software package. The curves were of the form:

$$y = ae^{-bx}$$

where a and b are constants.

When the stimulating voltage (V) equals the rheobasic voltage (V_o), $V/Vo = 1$.

Using the equation above, chronaxie = x when $y = 2$ because chronaxie is the time when the stimulus is twice rheobasic voltage.

Therefore, solving for x when $y = 2$:

$$y = ae^{-bx}$$

$$2 = ae^{-bx}$$

$$-bx = \ln(2/a)$$

$$x = -\ln(2/a)/b$$

From the semilogarithmic graph where $y = 2$, $x = \log_{10}$ chronaxie:

Therefore:

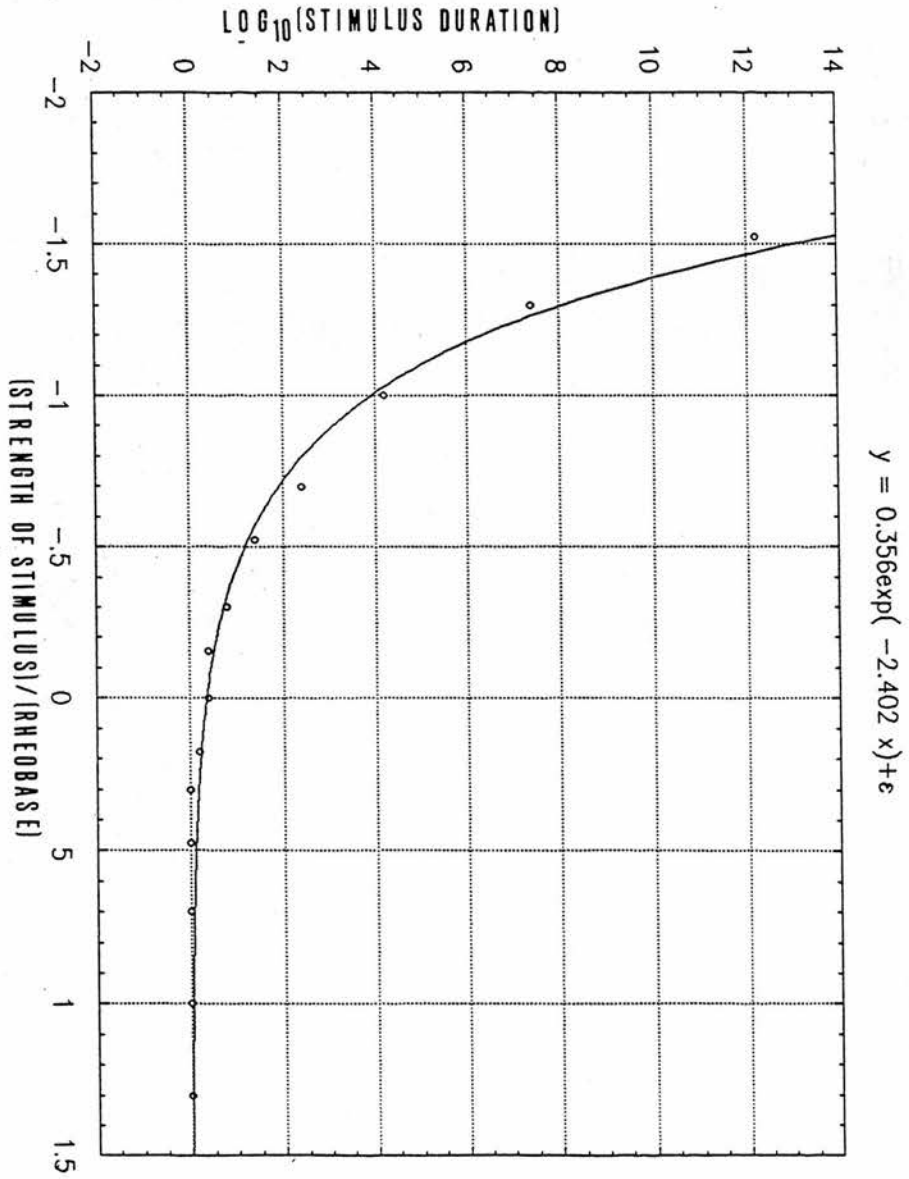
$$\text{Chronaxie} = \text{antilog } x/ \text{ ms}$$

Values of chronaxie for each rat were recorded.

4.2.7 Removal and Processing of Nerve.

After the electrophysiological assessment had been completed the animals were taken back to the operating table. The sciatic nerves were followed distally to their bifurcation into tibial and common peroneal branches. The common peroneal branch of each sciatic nerve was removed immediately distal to the bifurcation point. The removed nerves were then laid straight on pieces of card and put into test tubes containing 4% glutaraldehyde fixative in 0.1 M cacodylate buffer. After one hour the nerves were cut transversely into lengths of 1 mm and put into fresh fixative for another hour. The nerve was subsequently processed as in chapter 2.7. Sections of approximately 0.7 μm were cut using a Reichert OMU3 ultramicrotome (Reichert viz. Leica, Cambridge, U.K.).

Figure 4.7 Graph of a strength-duration curve. The values of $\log_{10}$(stimulus duration/ ms) plotted against (strength of stimulus/ V)/ (rheobase/ V)



4.2.8 Systematic Randomized Sampling.

When initiating a study involving sampling, it is of paramount importance that the scheme chosen is properly randomized. By definition, the samples used will be unbiased.

Bias is an indication of how the calculated value differs in magnitude and direction from the true value. Bias is totally independent of sample size (Mayhew, 1990). Bias arises from the intrinsic problems of the samples themselves e.g. shrinkage, obliquety of nerve sections; bias can also arise from inappropriate criteria used for sample selection. Biased samples can lead to false results evaluated from the application of statistics.

Efficiency also has to be considered before initiating any study. Efficiency is an indication of low random error (or precision) per unit cost (e.g. time). The larger the amount of time spent counting, the lower the efficiency. If the sample is small, there is a greater chance of error and the scheme is less efficient.

Mayhew (1990) stated that "It is not advisable or sensible to cut down on the number of animals simply in order to spend more time on sampling fibres within animals and on measuring those fibres very precisely". Considering the amount of material to be analysed, a sampling scheme was employed for counting fibres. The method of sampling chosen for counting nerve fibres was systematic random sampling. Unlike simple random sampling, the aforementioned method allows even sampling from all areas of the section and thereby increases efficiency. A similar method of sampling was used to measure axon diameter and fibre diameter of a representative sample of myelinated fibres (see section 4.2.9).

In order to obtain unbiased samples for counting, the sections to be counted had to be properly randomized. The orientation of sections on the

glass slides was totally random and ensured that every area of the section had the same independent chance of being selected. Specimen areas to be counted or 'windows' were selected by placing a grid of squares over the section (see below) and a ratio of the total number of squares was counted. Mayhew (1990) suggested a selection of 10 windows with approximately 20 fibres in each.

Calculation of grid size.

The number of myelinated fibres in the common peroneal nerve is approximately 2000 (Myles & Glasby, 1991). The number of grid squares required in order for each to contain approximately 20 fibres was calculated.

The approximate number of squares required = 2000/ 20 = 100

Mayhew suggested that 10 windows were counted.

The ratio of window to be counted = 10/ 100 = 1/ 10

The first window to be counted was chosen at random, by picking a number between 1 and 10. After counting the fibres within this window, the fibres in every tenth window were counted . The fibres counted were the ones whose centres fell within the grid squares. This avoids the common selection bias which results from counting fibres which fall completely within the grid squares; such a system is biased towards small fibres.

After completion of sampling, the total number of fibres was calculated using the fractionator method (Mayhew, 1992), (see below).

The number of windows counted divided by the total number of grid squares falling on the section gives a fraction of the section calculated

$$= 1/f$$

If the total number of fibres counted $= n$

The total number of fibres in the nerve $= n \times f$

4.2.9 Morphometric Assessment.

The VIDS III (Analytical Measuring Systems, Saffron Walden, U.K.) image analysis system is a high resolution, semi-automatic, image analysis system. Nerve sections were viewed under a microscope and were imaged by a high-performance television camera and displayed on a high resolution colour monitor. The monitor was linked to a digitizing tablet with a cursor. Current passes through a grid of wires in the tablet and movement of the cursor over the tablet induces a current in a coil situated at the focus of the cursor. The movement of the cursor over the tablet thereby causes the simultaneous movement of a cursor on the monitor screen.

The VIDS III system was used to 'mark' points on the screen and data handling was carried out by an IBM XT computer interfaced to the VIDS III system. In this way numbers of fibres were counted. The VIDS III was also used to measure lengths. The lengths measured were fibre and axon diameter of each neuron. To avoid bias arising from making measurements across obliquely cut sections, diameters were measured across the minor axes of elliptical profiles (Mayhew, 1990).

A glass slide bearing the semithin, 0.7 μm, nerve sections was placed under the microscope and the sections were viewed under oil. A section

was displayed on the monitor at a magnification of X100. The system was calibrated to correspond with the magnification at which the section was displayed on the monitor; the movement of the cursor was recorded and the distance in pixels was converted to microns by the VIDS III system.

Grid squares were photocopied onto transparencies. The size of the squares was such that they covered approximately 20 fibres of a nerve section when it was displayed on the monitor at a magnification of X100. The transparencies with grid squares photocopied onto them, were then placed over the monitor screen. The glass slide could be moved on the microscope stage so as to display one part of the nerve section, on the monitor, at a time. In this way each part of the section was systematically viewed and fell within one of the squares (or windows) of the transparency overlay. Fibres with their centres lying in every tenth grid square were counted as described above.

After counting had been completed, the total number of fibres was estimated using the fractionator method (see above).

The VIDS III system was used to measure axon and fibre diameters. Pairs of values, of axon and fibre diameter, were recorded for those fibres whose centres lay within the windows chosen to be sampled (see above). After completing the measurements, G-ratio was calculated by dividing values of axon diameter by the corresponding value of fibre diameter. G-ratio is indicative of nerve fibre maturation (Glasby *et al.*, 1986). When nerve fibres regenerate, they do not regain control values of axon diameter (Gutmann & Sanders, 1943) and myelination of smaller diameter axons is less. A measure of the myelin thickness alone, in regenerated fibres, would be expected to produce significantly different results from control nerve. Therefore, a much better indication of maturation is to measure the

relationship between axon and fibre diameters and compare this relationship with control nerve; hence, G-ratio was calculated in all groups.

4.3 Results.

4.3.1 Statistical Interpretation of Results.

The application of statistics to raw data allows the information obtained from experimentation to be interpreted in a way that is easily understood. The importance of choosing a statistical test that is appropriate for the experimental data and thus will provide meaningful answers to questions posed, cannot be overstressed. It should also be born in mind that statistics can never prove or disprove a hypothesis but merely give the probability that a result is significant.

Before statistical analysis can take place, one must first consider how the data are distributed. When possible, parametric tests were used in the work presented here because they employ the use of real values obtained from experiments. Non-parametric tests involve a process of ranking, where the true values obtained from experimentation are assigned a number depending on their position relative to other data points. Sensitivity is thus often lost when non-parametric tests are used. Before using parametric statistical analysis, data have to be normally distributed.

The normal distribution.

The curve of a normal or Gaussian distribution is symmetrical about the mean and bell-shaped. Normal distributions can vary in their shape. The standard deviation shows the spread of results about the mean and tends to be smaller for larger samples and *vice versa*. Samples with small standard deviations have a more 'peaked' bell-shaped curve than samples with a large standard deviation where the bell-shape is shorter and wider.

The data obtained for this thesis were tested for normality before any statistical tests were applied. Testing for normality was performed by plotting frequency distributions and histograms of sets of values and then normal distribution curves were fitted (Statistica, U.S.A.) and/or making normal plots. The latter process is described below. It is particularly important to test for a normal distribution when dealing with ratios. This is because ratios are intrinsically unstable by their very nature i.e. dividing one number by another number. Even if both of the two variables which constitute the ratio are normally distributed, the ratio itself might not be similarly distributed and may need a transformation. Transformations e.g. logarithmic or power transformations involve the transformation of the data points to a new set of values which are normally distributed.

The normal plot.

Suppose there are 50 individual values of a measured variable obtained from 10 groups of rats (i.e. 5 rats in each group), a combined mean of that variable is found by pooling the data, adding the values together and dividing by 50. The residuals of a variable are found by subtracting the combined mean from each measured value of the variable. The plot of residuals plotted against the mean for each group of rats shows the spread of the residuals for each group. The plot shows whether the variance changes with each group mean. A test for normality is the normal plot for the residuals: i.e. the residuals against their expected value when the distribution is normal. If the data are normally distributed, a straight line should be obtained for normal errors. A half normal plot was also made. This is used to detect outliers and involves plotting the absolute residuals, i.e. the sign of the residuals (–/+) is ignored, against a half normal distribution i.e. the expected values of the absolute residuals (which are

always >1). When outliers were detected, the values were rejected and the same process as above was repeated. Normal and half normal plots were produced for all the data presented in this thesis.

The standard normal distribution.

Normally distributed data can be described in terms of numbers of standard deviations from the mean and is called the standard normal distribution (SND). The mean of the SND is zero and the standard deviation is unity. The SND is produced from normally distributed data in the population using the equation:

$$SND,\ z = (x\text{-}u)/\ STD$$

where: x = variable

u = population mean

STD = standard deviation of the population

The SND curve is also a bell-shaped curve and 5% of the area under the curve lies outside $z = \pm 1.96$. A sample mean which lies beyond ± 1.96 has less than 5% probability of belonging to the same population. 1.96 is said to be the 5% percentage point of the normal distribution.

The mean of a sample is unlikely to be the same as the population mean. In fact, the means of different samples are distributed normally about the population mean. The standard deviation of this distribution is called the standard error of the mean (SEM) and is calculated from the equation:

$$SEM = SD/\sqrt{n}$$

where: SD = standard deviation of the sample

n = sample size

Significance testing.

Before initiating a significance test a null hypothesis is described which asserts that there is no real difference between different samples. In this thesis, the null hypothesis asserts that there is no real difference in recovery of peripheral nerve after the different types of nerve injury and repair (chapter 2.5). A further null hypothesis asserts that diabetes makes no real difference to the recovery of peripheral nerve after nerve injury and repair.

Parametric tests.

a) The F test for variance.

If the experimental data had been shown to be normally distributed (see above) an analysis of variance or F test was carried out on all 10 samples i.e. control, crush, nerve-to-nerve suture, nerve graft and muscle grafted groups in both the diabetic and non-diabetic states. The F test is a test of variance that can be applied to all samples simultaneously. It works on the principle that if the samples are from the same population, the variation in the data attributable to the differences between the group means can be accounted for by the variation attributable to the differences between

individuals (Kirkwood, 1991). A value of F was calculated using Genstat 5 (Rothamstead Experimental Station, Harpenden, U.K.) and the significance level was found from a table of values of percentage points for the F distribution. A result was considered significant when $p \leq 0.05$ indicating that the samples were probably not from the same population and the null hypothesis should be rejected. A significant result from the F test does not indicate which of the samples are unlikely to be from the same population. In order to find out which of the samples differed from the others, the two-sided Student's t test was applied to the difference between the means of combinations of samples (see below). The advantage of doing an F test is that only one test is required for all 10 samples. Doing multiple t tests increases the chance of type I error; this occurs when a true null hypothesis is wrongly rejected. At the 5% level of significance there is a probability that 1 in 20 of tests will yield a significant result. An F test indicates whether samples are significantly different from each other. In the work presented in this thesis where the null hypothesis for the F test was accepted, t tests were not carried out on the data. As a consequence of performing the F test, the sum of squares due to differences between the observations within each group is calculated; this value divided by the number of degrees of freedom (d.f.) gives the combined variance (SD^2) of all the samples:

$$SD^2 = \text{sum of } (x\text{-}m)^2 / \text{ d.f.}$$

where: x = an observation

m = mean of all the observations

$$\text{d.f.} = (n_1 + n_2 + \ldots\ldots + n_y) - y$$

The combined variance of all the samples is used in the Student's t test described below.

b) The Student's two-sided t test.

A two-sided test allows for departure from the null hypothesis in either direction e.g. the recovery of conduction velocity after muscle grafting might be the same as, better or worse than recovery after nerve grafting.

Any t test involves dividing something by the standard error. A t test comparing two means assesses the probability that there is no difference between the two sample means i.e. the two samples belong to the same population.

The same principle for producing the SND (see above) is used to produce the t distribution i.e. the t distribution is described by numbers of standard errors of the mean from the population mean in contrast to the SND which is described by the numbers of standard deviations from the population mean. The t distribution is described by:

$$t = (u-m)/ SEM, \text{ d.f. } = n-1$$

where: u = population mean

m = sample mean

$$SEM = (SD/ \sqrt{n})$$

The exact shape of the distribution depends on the number of degrees of freedom (d.f.) of the sample standard deviation (SD). The fewer the d.f. there are, the more spread out the t distribution is. Similarly to SND, percentage points can be determined, however, the 5% percentage point is

dependent on the number of d.f. e.g. the 5% percentage point for a sample size of 8 with 7 d.f. is 2.36 whereas the same percentage point for a sample size of 5 and 4 d.f. is 2.78.

The equation for the t distribution (above) is used for comparing a sample mean with a population mean. In the work presented in this thesis, the population mean was not known and the sample size was never big enough (i.e. $n > 60$) to enable the use of this equation. Instead a variation of this equation is used when dealing with small independent samples. The equation used in the work presented in this thesis describes the distribution of the difference in sample means (m_1-m_2). The mean of this distribution is the difference between the two population means (u_1-u_2), which, if the null hypothesis is correct, is 0. The t distribution is produced by dividing the difference in sample means by the standard error. The standard error is calculated from the standard deviation i.e. the square root of the variance (see above) where:

$$SEM = SD \times \sqrt{(1/n_1 + 1/n_2)}$$

The data from all 10 samples were pooled to give a better estimate of variance (see above); this in turn, because of the higher number of d.f. produces a more powerful t test when differences in means are compared. Underlying this method is the presumption that the distribution of all samples are normal and have the same variation. These were both confirmed as described above. The t values were calculated from the following equation:

$$t = (m_1 - m_2) / \text{SEM}$$

where: m_1 = mean of sample 1

m_2 = mean of sample 2

When using t tests to compare many combinations of means of samples, the value of t which gives significance levels of 5%, 1% etc. has to be adjusted to allow for purely chance significance. Type 1 error is described above. Scheffé's multiple confidence intervals were used. The confidence intervals (c.i.) are calculated as follows:

Let $\underline{s}$ = number of samples i.e. 10

c.i. at 5% level = $\sqrt{((\underline{s}-1) \times F)}$

where: F is found from a table of percentage points for the F distribution (0.05; $\underline{s}$-1; n-$\underline{s}$+1).

4.3.2 Results from Nerve Conduction Velocity Experiments.

8 of the 10 groups were composed of 5 rats each. The eight groups were the control, nerve crush, nerve-to-nerve suture and nerve grafted groups in the diabetic and non-diabetic states. The remaining 2 groups i.e. diabetic muscle grafted animals and non-diabetic muscle grafted animals each contained 6 animals. A table of the values of the conduction velocity of the fastest fibres in the sciatic nerve of each rat is shown in appendix 7. The values of the mean, standard deviation, standard error and the coefficient of variation of conduction velocity for each group of non-diabetic animals are displayed in table 4.1, and a bar chart showing mean conduction velocity for all of the non-diabetic groups is displayed in figure 4.8. The equivalents for the diabetic animals are displayed in table 4.2 and figure 4.9. It can be seen that the mean conduction velocity is lower in all diabetic groups compared with the corresponding non-diabetic group. Also, the degree of recovery of conduction velocity varied inversely with the severity of the nerve injury in most cases; the exception being the non-diabetic nerve-to-nerve suture group.

Table 4.1 Values of the mean, standard deviation, standard error of the mean and coefficient of variation of the left (operated) sciatic nerve conduction velocity in non-diabetic animals

RAT GROUP	n	m /m s^{-1}	SD	SEM	c.v.
C	5	44.82	3.45	1.54	7.70
CR	5	32.68	4.86	2.17	14.86
NN	5	41.04	8.52	3.81	20.76
NG	5	21.65	10.17	4.55	46.98
MG	6	12.34	3.39	1.38	27.46

Figure 4.8 Bar chart to show the mean conduction velocity of each of the non-diabetic groups of animals (n = 5 for each group)

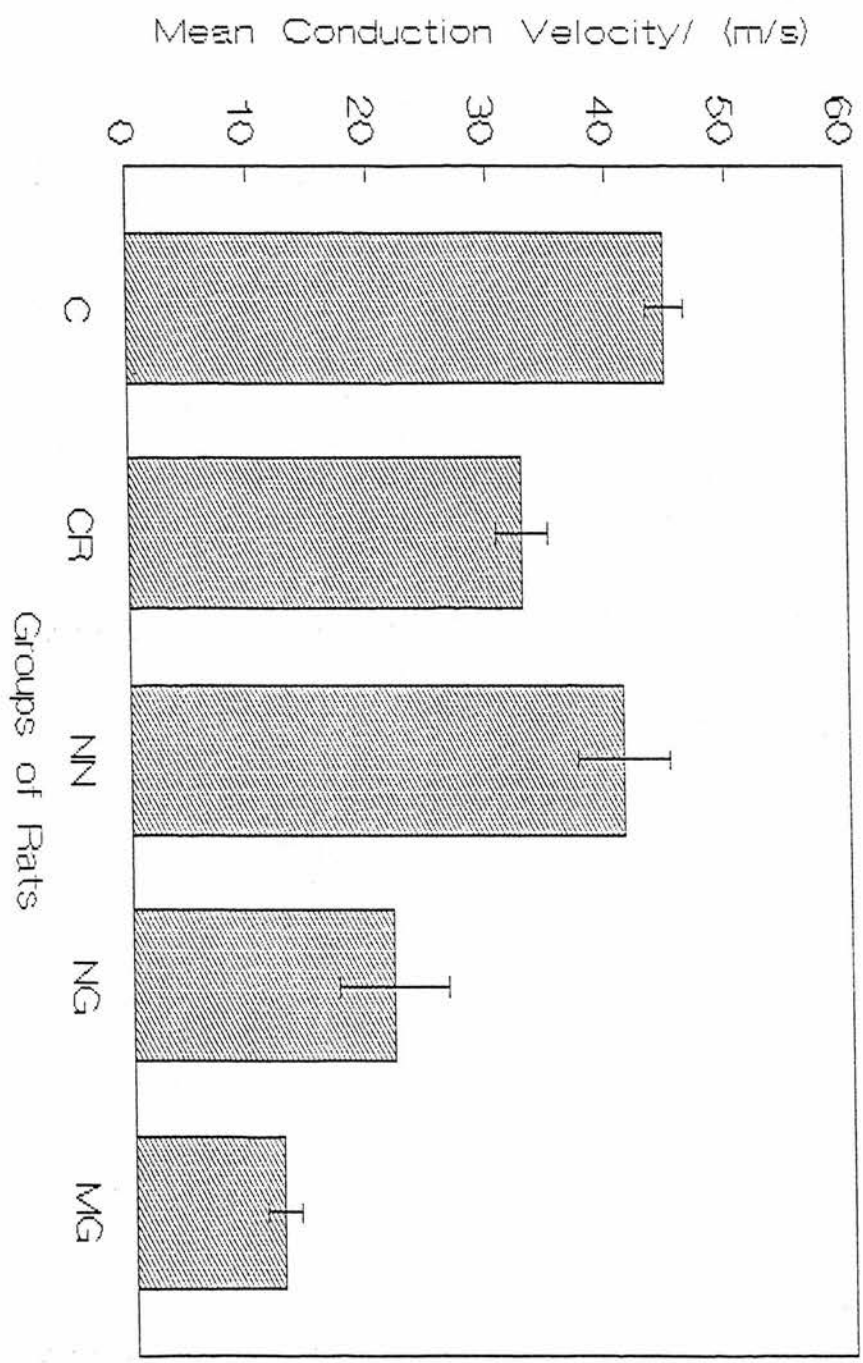


Figure 4.9 Bar chart to show the mean conduction velocity of each of the diabetic groups of animals (n = 5 for each group)

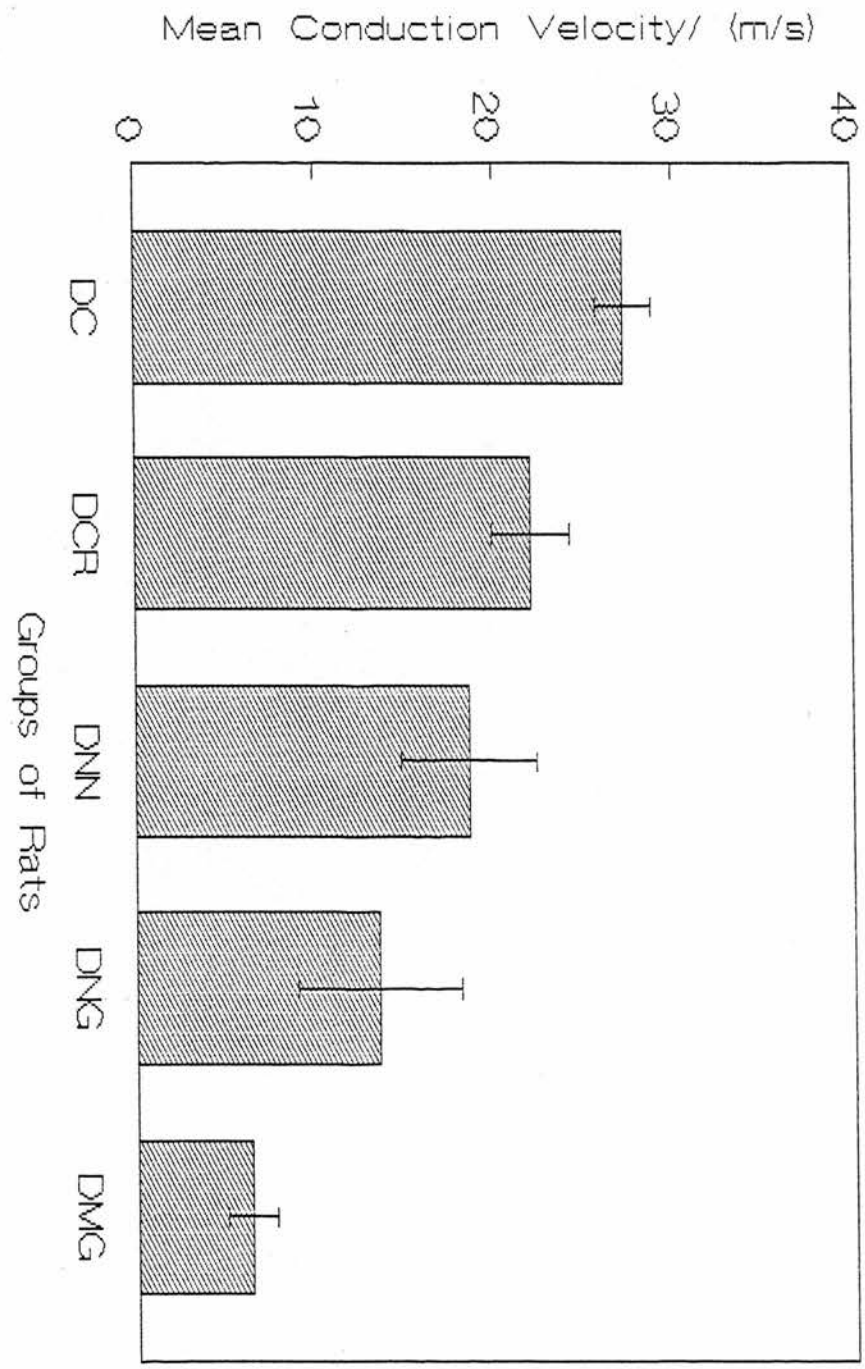


Table 4.2 Values of the mean, standard deviation, standard error of the mean and coefficient of variation of the left (operated) sciatic nerve conduction velocity in diabetic animals

RAT GROUP	n	m /m s^{-1}	SD	SEM	c.v.
DC	5	27.26	8.36	3.74	30.68
DCR	5	22.00	3.77	1.69	17.16
DNN	5	18.52	1.12	0.50	6.02
DNG	5	13.47	4.87	2.18	36.18
DMG	6	6.32	2.66	1.08	42.08

With sample sizes of n = 5 or n = 6 it is not possible to produce frequency distributions for each sample, therefore, a normal plot was made (see above) for the residuals of conduction velocity of the fastest fibres of the sciatic nerve. Frequency distributions were constructed, however, for the values of conduction velocity which were compiled from the control sides of all groups in each of the metabolic states and frequency histograms were then plotted (appendices 8 and 9). The observed frequency of values which fell between boundaries of 5 ms^{-1}, from 30-60 ms^{-1}, were calculated for the non-diabetic animals (appendix 8). Similar boundaries of 5 ms^{-1} were used for the diabetic animals but allowing for the fact that the conduction velocities were much lower in the diabetic rats, the range extended from 10-55 ms^{-1} (appendix 9). From the construction of normal plots and frequency histograms it was found that the data of conduction velocity were normally distributed. This finding was a justification for the use of parametric tests (in fact, the F test and the Student's two-tailed t test for the difference between two means were used). Previous authors have used parametric tests on similar data (Cragg & Thomas, 1964; Thomas *et al.* 1981). However, when using the t test for comparing the difference between two means, there lies the assumption that the standard deviations are similar (see above). In many situations it is reasonable to assume equality of the

standard deviations (Kirkwood, 1991). From tables 4.1 and 4.2, which show the values of the mean, standard deviation, standard error and the coefficient of variation of the conduction velocity of the fastest fibres of the sciatic nerve, for the non-diabetic and diabetic groups respectively, it is apparent that the standard deviation of the conduction velocity varies quite significantly between groups. A difference in the size of the standard deviation between samples is significant when one sample has a standard deviation twice or more that of the other (Kirkwood, 1991). For this reason a ratio was taken of the value of conduction velocity of the left sciatic nerve divided by the value of the conduction velocity of the right sciatic nerve, for each rat (CV ratio). The values of CV ratio are displayed in appendix 7. The CV ratio is the conduction velocity of the fastest fibres in the sciatic nerve expressed as a fraction of the expected value of conduction velocity had no operation taken place. The use of the ratio assumes that left and right sciatic nerve conduction velocities are of a similar size in control animals. The mean values of CV ratio for the non-diabetic control group and diabetic control group approximated to 1 ($m = 1.11$, $SD = 0.21$ and $m = 0.91$, $SD = 0.12$, respectively), verifying a relationship between the values of conduction velocity of the two sides within an individual. Cragg and Thomas (1961) demonstrated that the conduction velocity of the peroneal nerves in rabbits, were very similar on both sides and have, in fact, used the values of conduction velocity on the unoperated sides as controls (Cragg & Thomas, 1964).

It was verified that the values of CV ratio were normally distributed using the normal plot method. Tables 4.3 and 4.4 show the values of the mean, standard deviation, standard error and the coefficient of variation of the CV ratio for non-diabetic and diabetic animals respectively. From these tables it is apparent that the values of standard deviation of CV ratio, for

each animal group, are more similar than the values of standard deviation of conduction velocity. Owing to the closeness in size of the standard deviations and the normal distribution of the CV ratio, it was decided that the CV ratio should be used in the statistical tests. There are considerable advantages in using the conduction velocity ratio. When recording the conduction velocity of peripheral nerve in the laboratory, it is well known that environmental factors can affect the results considerably. For example Birren and Wall (1956) found that a change of $1^{o}C$ in temperature, over the range of 20 to $40^{o}C$, will affect conduction velocity by approximately 12 m s^{-1}. Although attempts are made to maintain consistent conditions for each experiment, this can not be guaranteed. Use of the conduction velocity ratio in statistical tests as opposed to the use of absolute conduction velocity values may be better because of the variation in the latter, arising from environmental fluctuations, and its consequent larger standard deviation. Obviously, if the experimental design is good, use of the CV ratio will not have any great advantage in this respect. It is also known that the age of the animal affects peripheral nerve conduction velocity and that it increases up to the age of 9 months (Sharma & Thomas, 1974). In the experiments presented in this thesis all of the animls were of the same age but the CV ratio would reduce the variation caused by such a factor. Individual variation in conduction velocity in rats of the same age, sex, breed *etc.* cannot be dealt with by good experimental design. However, by expressing the conduction velocity of one side as a ratio of the other, the natural variation in conduction velocity from one animal to another is overcome.

Table 4.3 Values of the mean, standard deviation, standard error of the mean and coefficient of variation of the CV ratio in non-diabetic animals

RAT GROUP	n	m	SD	SEM	c.v.
C	5	1.11	0.21	0.09	18.71
CR	5	0.74	0.16	0.07	22.15
NN	5	0.83	0.12	0.05	14.48
NG	5	0.49	0.23	0.10	48.16
MG	6	0.29	0.10	0.04	34.23

Table 4.4 Values of the mean, standard deviation, standard error of the mean and coefficient of variation of CV ratio in diabetic animals

RAT GROUP	n	m	SD	SEM	c.v.
DC	5	0.91	0.12	0.05	12.97
DCR	5	0.74	0.08	0.04	11.03
DNN	5	0.82	0.16	0.07	18.94
DNG	5	0.52	0.10	0.05	19.93
DMG	6	0.28	0.12	0.05	43.02

Importantly, the CV ratio is necessary when comparing the recovery of conduction velocity between diabetic and non-diabetic groups. This is because the conduction velocity of peripheral nerve in diabetic animals is already significantly lower than that of the diabetic animals ($p < 0.01$, for non-diabetic controls *v* diabetic controls; Student's t test). The significant difference between diabetic and non-diabetic animals is due to the effect of diabetes on peripheral nerve conduction velocity and not an indication of poorer recovery of conduction velocity in diabetic nerve after nerve injury and repair. By expressing the values of the recovered conduction velocity as a ratio of the conducion velocity on the unoperated side, the slowing effect of diabetes on peripheral nerve conduction velocity regardless of injury and repair is overcome.

By expressing the mean value of the left operated sides as a percentage of the mean values of conduction velocity on the right unoperated sides, it was possible to compare the recovery of matched diabetic and non-diabetic groups. The non-diabetic crushed nerve regained 72.70% of the control mean. This was very similar to the diabetic crushed nerve which regained 74.59%. The values of percentage recovery of conduction velocity in the non-diabetic and diabetic nerve-to-nerve suture groups were also similar: 83.23% and 79.92% respectively. Poorer recovery was found after nerve grafting; 48.33% in the non-diabetic group and 52.00% in the diabetic group. The muscle grafted groups both gave similarly poor results; 28.41% in the non-diabetic group and 26.15% in the diabetic group.

Results from significance tests on the values of CV ratio.

a) F test for variance.

From the F test for variance performed on the data obtained from all of the animals, the value of F was found to be = 14.82, d.f. = 9, 51. This result was highly significant ($p < 0.01$) suggesting that the null hypothesis should be rejected as the samples were unlikely to be from the same population.

b) Student's t tests on the means of CV ratio.

Non-diabetic rats.

Table 4.5 shows the values of t and the significance levels (at 5% or 1% levels) for t tests performed on the means of the CV ratio for each non-diabetic group, as compared with each other. The recovery of conduction velocity after muscle grafting was particularly poor ($p < 0.01$). The recovery of conduction velocity after nerve crush and nerve graft was

also poor, as compared to controls ($p < 0.05$ and $p < 0.01$, respectively). Although the mean CV ratio of the nerve-to-nerve suture group ($m = 0.74$, $SD = 0.16$, $n = 5$) was less than the mean CV ratio of the controls ($m = 1.11$, $SD = 0.21$, $n = 5$), they were not significantly different at the 5% level.

Table 4.5 Table of t values and significance levels for the values of the ratio of CV ratio in non-diabetic animals (Student's t test)

	C	CR	NN	NG	MG
C	–	t=3.56 p<0.05	t=2.70 NS	t=6.00 p<0.01	t=8.22 p<0.01
CR	–	–	t=-0.85 NS	t=2.43 NS	t=4.51 p<0.01
NN	–	–	–	t=3.29 p<0.05	t=5.40 p<0.01
NG	–	–	–	–	t=1.97 NS
MG	–	–	–	–	–

The recovery of conduction velocity of the nerve-to-nerve suture group was actually better than the recovery of the nerve crush group. The mean CV ratio of the nerve-to-nerve-suture group was 0.83 ($SD = 0.12$, $n = 5$) as compared with a mean of 0.74 for the nerve crush group; these means were not significantly different at the 5% level. The recovery of conduction velocity after nerve grafting was not significantly different from the recovery after crushing the nerve ($p > 0.05$), however, the recovery of conduction velocity after muscle grafting was significantly worse than after a nerve crush injury ($p < 0.01$).

As stated above, the nerve-to-nerve suture group recovered a greater mean conduction velocity than the nerve crush group, although the means

of the CV ratios were not significantly different ($p > 0.05$). The mean of the CV ratio for the nerve-to-nerve suture group was significantly different from both the nerve graft and muscle graft groups ($p < 0.05$ and $p < 0.01$, respectively).

The mean of CV ratio for the muscle grafted animals was much lower than the nerve grafted animals, $m = 0.29$ ($SD = 0.10$, $n = 6$) as compared with $m = 0.49$ ($SD = 0.23$, $n = 5$), the means were not significantly different at the 5% level.

In summary, the recovery of conduction velocity was increasingly poor with increasing severity of nerve injury with the exception of the nerve-to-nerve suture group which recovered a greater mean CV ratio than the nerve crush group. This is discussed in chapter 4.4.

Diabetic rats.

Table 4.6 shows the values of t and the significance levels for t tests performed on the means of CV ratio of each diabetic group, as compared with each other. The pattern of significance for the diabetic animals can be seen to be similar to the pattern of significance for the non-diabetic animals, displayed in table 4.5. The mean CV ratio of the nerve crush and nerve-to-nerve-suture groups ($m = 0.74$, $SD = 0.08$, $n = 5$ and $m = 0.82$, $SD = 0.16$, $n = 5$, respectively) was smaller than the mean of the control animals ($m = 0.91$, $SD = 0.12$, $n = 5$), however, they were not significantly different from each other at the 5% level. The mean CV ratio of the nerve graft and muscle groups was significantly smaller than the control group ($p < 0.01$).

Table 4.6 Table of t values and significance levels for the values of the ratio of CV ratio in diabetic animals (Student's t test)

	DC	DCR	DNN	DNG	DMG
DC	_	t=1.65 NS	t=0.88 NS	t=3.78 p<0.01	t=6.37 p<0.01
DCR	_	_	t=-0.77 NS	t=2.13 NS	t=4.65 p<0.01
DNN	_	_	_	t=2.90 NS	t=5.45 p<0.01
DNG	_	_	_	_	t=2.42 NS
DMG	_	_	_	_	_

Similarly to the non-diabetic animals, the mean CV ratio of the nerve-to-nerve suture group was bigger than the mean of the nerve crush group, m = 0.82 (SD = 0.16, n = 5) as compared with m = 0.74 (SD = 0.08, n = 5), although they were not significantly different ($p > 0.05$). The recovery of conduction velocity of the nerve crush group was not significantly better than the nerve graft group ($p > 0.05$) but the nerve crush group did recover conduction velocity significantly better than the muscle graft group ($p < 0.01$).

The mean CV ratio of the muscle grafted animals was significantly smaller than the nerve-to-nerve suture group ($p < 0.01$). The mean CV ratio of the nerve graft group was smaller than the nerve-to-nerve suture group, though not significantly so ($p > 0.05$).

The recovery of conduction velocity after muscle grafting was significantly worse than the control, nerve crush and nerve-to-nerve suture groups ($p < 0.01$). Although the mean CV ratio of the muscle graft group was much smaller than the nerve graft group, m = 0.28 (SD = 0.12,

n = 6), as compared with m = 0.52 (SD = 0.10, n = 5), the means were not significantly different from each other at the 5% level.

In summary, the recovery of conduction velocity was increasingly poor with increasing severity of nerve injury. The only exception was that of the nerve-to-nerve suture group, which recovered a greater mean CV ratio than the nerve crush group, though the groups were not significantly different at the 5% level.

Diabetic *v* non-diabetic rats.

Table 4.7 shows the t values and significance levels for t tests performed on like groups from the diabetic and non-diabetic populations.

Table 4.7 Table of t values and significance levels for the values of CV ratio in non-diabetic v diabetic animals (Student's t test)

	C	CR	NN	NG	MG
DC	t=-1.862 NS	–	–	–	–
DCR	–	t=0.05 NS	–	–	–
DNN	–	–	t=-0.04 NS	–	–
DNG	–	–	–	t=0.35 NS	–
DMG	–	–	–	–	t=-0.10 NS

None of the pairs of samples was significantly different from any other ($p > 0.05$) indicating that recovery of conduction velocity was unaffected by the diabetic state.

4.3.3 Results from Nerve Excitability Experiments.

Each group of rats consisted of 5 animals except for the diabetic muscle graft group which consisted of 6 animals. The values of chronaxie obtained from the left and right sciatic nerves of each animal are displayed in appendix 10. Table 4.8 and 4.9 show the values of the mean , standard deviation, standard error of the mean and coefficient of variation of chronaxie, recorded from the left sciatic nerve, for the non-diabetic and diabetic groups, respectively. Tables 4.10 and 4.11 show the equivalent information from the results of chronaxie, obtained from the right (unoperated) sciatic nerve of the non-diabetic and diabetic rats. A plot was made of the residuals against their fitted values (see 'normal plot' above). From this plot it was found that the variance was similar for each group. Normal and half-normal plots were constructed. From such plots it is possible to detect outliers, in fact, one outlier not belonging to the normal distribution of the rest of the data was found. This value was 0.74 ms and was recorded from one of the diabetic muscle grafted animals. This value was ignored and the process of producing normal and half-normal plots was repeated. Straight lines were obtained, thus indicating a normal distribution of values and providing justification for the use of parametric tests. The value not corresponding to the normal distribution was not used in the statistical tests.

Table 4.8 Values of the mean, standard deviation, standard error of the mean and coefficient of variation of chronaxie on the left (operated) sides in non-diabetic animals

RAT GROUP	n	m /ms	SD	SEM	c.v.
C	5	0.29	0.13	0.06	45.43
CR	5	0.33	0.12	0.05	35.65
NN	5	0.27	0.07	0.03	23.87
NG	5	0.22	0.10	0.05	45.81
MG	5	0.25	0.12	0.05	49.57

Table 4.9 Values of the mean, standard deviation, standard error of the mean and coefficient of variation of chronaxie on the left (operated) sides in diabetic animals

RAT GROUP	n	m /ms	SD	SEM	c.v.
DC	5	0.19	0.05	0.02	24.20
DCR	5	0.19	0.07	0.03	36.87
DNN	5	0.24	0.10	0.05	41.91
DNG	5	0.19	0.07	0.03	39.03
DMG	6	0.37	0.19	0.08	51.02

Table 4.10 Values of the mean, standard deviation, standard error of the mean and coefficient of variation of chronaxie on the right (unoperated) sides in non-diabetic animals

RAT GROUP	n	m /ms	STD	SEM	c.v.
C	5	0.38	0.21	0.09	55.17
CR	5	0.40	0.16	0.07	40.99
NN	5	0.26	0.05	0.02	20.03
NG	5	0.32	0.31	0.14	96.16
MG	5	0.16	0.05	0.02	29.88

Table 4.11 Values of the mean, standard deviation, standard error of the mean and coefficient of variation of chronaxie on the (right) unoperated sides of diabetic animals

RAT GROUP	n	m /ms	STD	SEM	c.v.
DC	5	0.17	0.05	0.02	29.28
DCR	5	0.26	0.13	0.06	47.83
DNN	5	0.43	0.07	0.03	15.92
DNG	5	0.14	0.05	0.02	33.86
DMG	5	0.30	0.01	0.05	34.87

Results from significance tests on the values of chronaxie.

a) F test for variance.

The value of F obtained from performing the F test on the values of chronaxie from the left sciatic nerve was $F = 1.42$, d.f. $= 9, 49$. There was no significant difference between samples ($p > 0.05$) and the null hypothesis asserting that there is no difference in nerve excitability between samples was accepted.

4.3.4 Results from Nerve Morphometry.

The data on nerve morphometry were obtained from 50 animals; each group of animals consisted of 5 rats. Approximately 200 values of axon diameter, fibre diameter, myelin thickness and g-ratio were obtained from each animal. The mean of these values for each rat is displayed in appendix 11 and appendix 12. An example of a typical nerve section from each of the 10 groups is shown in plates 4.1-4.10.

Axon diameter.

The mean, standard deviation, standard error of the mean and the coefficient of variation of axon diameter for each group of non-diabetic animals are displayed in table 4.12 and the equivalents for the diabetic

groups of animals are displayed in table 4.13. Normal and half-normal plots constructed from the residuals of axon diameter showed that the values were normally distributed. The residuals of axon diameter plotted against the fitted values of the mean for each group showed that each sample had a similar variance. There were no outliers.

Table 4.12 Values of the mean, standard deviation, standard error of the mean and coefficient of variation of axon diameter in non-diabetic animals

RAT GROUP	n	m /μm	SD	SEM	c.v.
C	5	4.07	0.25	0.11	6.25
CR	5	2.83	0.15	0.07	5.18
NN	5	1.93	0.12	0.05	6.11
NG	5	1.74	0.11	0.05	6.23
MG	5	1.58	0.24	0.11	14.92

Table 4.13 Values of the mean , standard deviation, standard error of the mean and coefficient of variation of axon diameter in diabetic animals

RAT GROUP	n	m /μm	SD	SEM	c.v.
DC	5	3.86	0.23	0.1	5.85
DCR	5	2.11	0.29	0.13	13.86
DNN	5	1.98	0.36	0.16	17.91
DNG	5	1.67	0.23	0.1	14.04
DMG	5	1.45	0.2	0.09	14.11

Results from significance tests on the values of axon diameter.

a) F test for variance.

The value obtained for F from the results of axon diameter was $F = 68.36$, d.f. $= 9, 49$. This was highly significant ($p < 0.001$) indicating that the samples were not from the same population.

b) Student's t tests on the means of axon diameter.

Non-diabetic rats.

The recovery of axon diameter to sizes similar to those found in controls was never attained. In fact, recovery of axon diameter was increasingly poor with increasing severity of nerve injury.

Table 4.14 shows the values of t and the significance of the Student's t tests performed on the means of axon diameter obtained from the non-diabetic animals. The mean axon diameter of the control animals was 4.07 μm which was significantly larger than the recovered axon diameter of any other group ($p < 0.01$). The mean diameter of the nerve crush group was 2.83 μm and this was significantly larger than the mean axon diameters of the nerve-to-nerve suture, nerve graft and muscle graft groups ($p < 0.01$, in each case). The mean axon diameters of the nerve-to-nerve suture, nerve graft and muscle graft groups were 1.93, 1.74 and 1.58 μm, respectively. Although the mean axon diameter of the nerve-to-nerve suture group was larger than the nerve graft group and the muscle graft group, the means were not significantly different at the 5% level. The mean axon diameter of the muscle graft group was smaller than the nerve graft group but not significantly so ($p > 0.05$).

Table 4.14 Table of t values and significance levels for the values of axon diameter in non-diabetic animals (Student's t test)

	C	CR	NN	NG	MG
C	_	t=7.64 p<0.01	t=13.18 p<0.01	t=14.38 p<0.01	t=15.37 p<0.01
CR	_	_	t=5.54 p<0.01	t=6.74 p<0.01	t=7.72 p<0.01
NN	_	_	_	t=1.20 NS	t=2.19 NS
NG	_	_	_	_	t=0.98 NS
MG	_	_	_	_	_

Diabetic rats.

Similarly to the non-diabetic animals, the degree of recovery of axon diameter was inversely related to the severity of the injury. The diabetic crush group faired worse than the non-diabetic crush group when compared to the other injury types.

Table 4.15 shows the t values and the significance levels of t tests performed on the means of axon diameter obtained from the diabetic groups of animals. The mean value of axon diameter of the control group was 3.86 μm. This was significantly bigger than the mean value of axon diameter obtained from the other groups ($p < 0.01$). The mean axon diameter of the nerve crush group was 2.11 μm. Although larger than the mean diameters of the nerve-to-nerve suture and nerve graft groups, m = 1.98 and 1.67 μm, respectively, the mean of the nerve crush group was not significantly different from these means at the 5% level. The mean diameter of the muscle graft group was 1.45 μm which was significantly smaller than the recovered mean of axon diameter in the nerve crush group

($p < 0.05$). The mean axon diameter of the muscle graft group was also significantly smaller than the mean of the nerve-to-nerve suture group ($p < 0.05$). However, the means of the nerve graft and muscle graft groups were not significantly different ($p > 0.05$).

Table 4.15 Table of t values and significance levels for the values of axon diameter in diabetic animals (Student's t test)

	DC	DCR	DNN	DNG	DMG
DC	–	t=10.84 p<0.01	t=11.60 p<0.01	t=13.55 p<0.01	t=14.87 p<0.01
DCR	–	–	t=0.75 NS	t=2.71 NS	t=4.03 p<0.05
DNN	–	–	–	t=1.95 NS	t=3.27 p<0.05
DNG	–	–	–	–	t=1.32 NS
DMG	–	–	–	–	–

Diabetic *v* non-diabetic rats.

The means of axon diameter were smaller in the diabetic animals, except the diabetic nerve-to-nerve suture group whose mean axon diameter, m = 1.98 μm, was slightly larger than the mean obtained from the non-diabetic nerve-to-nerve suture group, m = 1.93 μm. Figure 4.10 is a bar-chart of the mean axon diameters of all groups. Table 4.16 shows the results of the t tests performed on the means of axon diameter from like groups in the diabetic and non-diabetic populations. The only significant result was that comparing the means of axon diameter from the two crush groups ($p < 0.01$).

Figure 4.10 Bar chart of the mean axon diameter of each group of animals (n = 5 for each group)

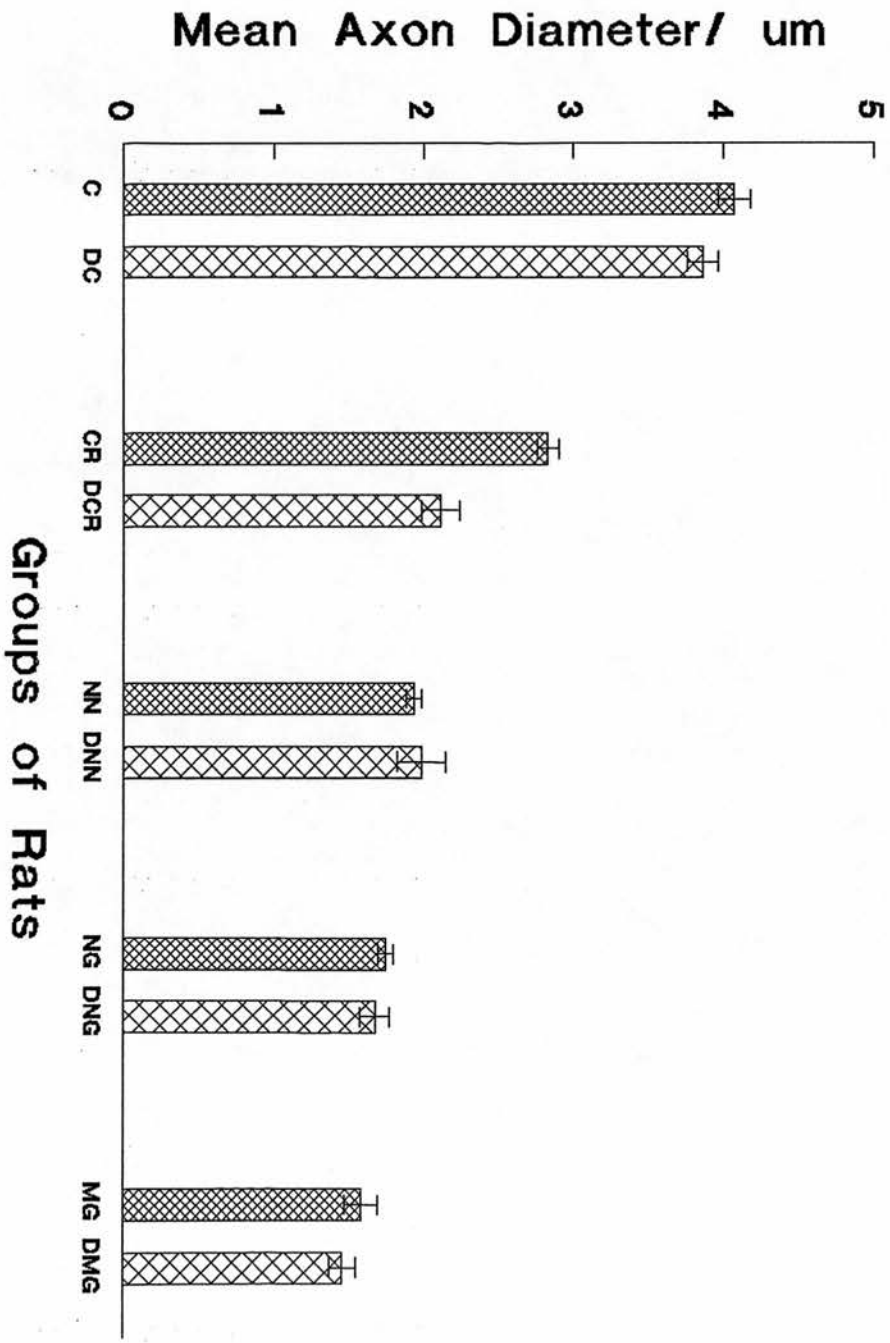


Table 4.16 Table of t values and significance levels for the values of axon diameter in non-diabetic v diabetic animals (Student's t test)

	C	CR	NN	NG	MG
DC	t=1.29 NS	–	–	–	–
DCR	–	t=4.49 p<0.01	–	–	–
DNN	–	–	t=0.29 NS	–	–
DNG	–	–	–	t=0.45 NS	–
DMG	–	–	–	–	t=0.79 NS

Fibre diameter.

Approximately 200 values of fibre diameter were obtained from each of 50 animals; there were 5 animals in each of the 10 groups. The mean fibre diameter for each animal is displayed in appendix 11 and appendix 12. Table 4.17 shows the values of the mean, standard deviation, standard error of the mean and the coefficient of variation of fibre diameter obtained from the different groups of non-diabetic animals. Table 4.18 shows the equivalent for the diabetic animals.

Table 4.17 Values of mean fibre diameter, standard deviation, standard error of the mean and coefficient of variation for non-diabetic animals

RAT GROUP	n	m /μm	SD	SEM	c.v.
C	5	7.35	0.13	0.06	1.78
CR	5	5.67	0.51	0.23	9.04
NN	5	4.02	0.34	0.15	8.58
NG	5	3.94	0.16	0.07	4.08
MG	5	3.78	0.24	0.11	6.30

Table 4.18 Values of mean fibre diameter, standard deviation, standard error of the mean and coefficient of variation for diabetic animals

RAT GROUP	n	m /μm	SD	SEM	c.v.
DC	5	6.99	0.33	0.15	4.66
DCR	5	4.63	0.27	0.12	5.85
DNN	5	4.14	0.42	0.19	10.18
DNG	5	3.94	0.42	0.19	10.72
DMG	5	3.38	0.21	0.09	6.11

A plot of the residuals of fibre diameter against the fitted values of the mean of each group, showed that the variance of each group was similar. Normal and half-normal plots provided confirmation of a normal distribution of values with no outliers.

Results from significance tests on the values of fibre diameter.

a) F test for variance.

A highly signifcant result, $F = 74.08$, d.f. $= 9, 49$, was obtained from the F test performed on the values of fibre diameter ($p < 0.01$). The null hypothesis asserting that there is no difference in the values of mean fibre diameter between different samples, was duly rejected.

b) Student's t tests on the means of fibre diameter.

Non-diabetic rats.

The pattern of significance between the means of fibre diameter from the different non-diabetic groups is identical to the pattern obtained for axon diameter. Fibre diameters of a similar size to control values were never obtained after nerve injury and repair. The recovery of fibre diameter was increasingly poor with increasing severity of the nerve injury.

Table 4.19 shows the t values and significance levels obtained from t tests performed on the means of fibre diameter from the non-diabetic animal

groups. The mean of the control group, m = 7.35 μm, was significantly bigger than any of the other means ($p < 0.01$). The mean fibre diameter obtained from the crush group was m = 5.67 μm. This mean was significantly bigger than the means obtained from the other three groups of injury and repair ($p < 0.01$) i.e. nerve-to-nerve suture, nerve graft and muscle graft groups. The mean fibre diameters for each of the nerve-to-nerve suture, nerve graft and muscle graft groups were not significantly different from each other ($p > 0.05$).

Table 4.19 Table of t values and significance levels for the values of fibre diameter in non-diabetic animals (Student's t test)

	C	CR	NN	NG	MG
C	_	t=7.31 p<0.01	t=14.48 p<0.01	t=14.81 p<0.01	t=15.50 p<0.01
CR	_	_	t=7.17 p<0.01	t=7.50 p<0.01	t=8.18 p<0.01
NN	_	_	_	t=0.33 NS	t=1.01 NS
NG	_	_	_	_	t=0.68 NS
MG	_	_	_	_	_

Diabetic rats.

Similarly to the non-diabetic animals, the recovery of mean sciatic nerve fibre diameter was poorer with increasing severity of injury.

Table 4.20 shows the t values and significance levels obtained from t tests performed on the means of fibre diameter from the diabetic animal groups. The muscle grafted animals showed the worst recovery of fibre diameter. The mean fibre diameter, m = 3.38 μm, was significantly smaller

than all of the other group means ($p < 0.01$) except the nerve graft group ($p < 0.05$). The mean of fibre diameter after nerve grafting was also small, $m = 3.94\ \mu m$, although not significantly smaller than the mean fibre diameter of the nerve-to-nerve suture group ($p > 0.05$), the mean of the nerve graft group was significantly smaller than both the crush group ($p < 0.05$) and the control group ($p < 0.01$). The nerve-to-nerve suture and nerve crush groups recovered mean fibre diameters of 4.14 and 4.63 μm, respectively. These means were not significantly different from each other ($p > 0.05$) but were significantly smaller than control values ($p < 0.01$).

Table 4.20 Table of t values and significance levels for the values of fibre diameter in diabetic animals (Student's t test)

	DC	DCR	DNN	DNG	DMG
DC	–	t=10.27 p<0.01	t=12.37 p<0.01	t=13.24 p<0.01	t=15.70 p<0.01
DCR	–	–	t=2.10 NS	t=2.97 p<0.05	t=5.43 p<0.01
DNN	–	–	–	t=0.87 NS	t=3.34 p<0.05
DNG	–	–	–	–	t=2.47 NS
DMG	–	–	–	–	–

Diabetic *v* non-diabetic rats.

The means of fibre diameter were smaller in the diabetic animals than the non-diabetic animals, except the diabetic nerve-to-nerve suture group whose mean fibre diameter, $m = 4.14\ \mu m$, was larger than the mean obtained from the non-diabetic nerve-to-nerve suture group, $m = 4.02\ \mu m$.

Figure 4.11 is a bar-chart of the mean fibre diameters of all groups. Table 4.21 shows the results of the t tests performed on the means of fibre diameter from like groups in the diabetic and non-diabetic populations. The only significant result was that from a comparison of the means of fibre diameter from the two crush groups ($p < 0.01$).

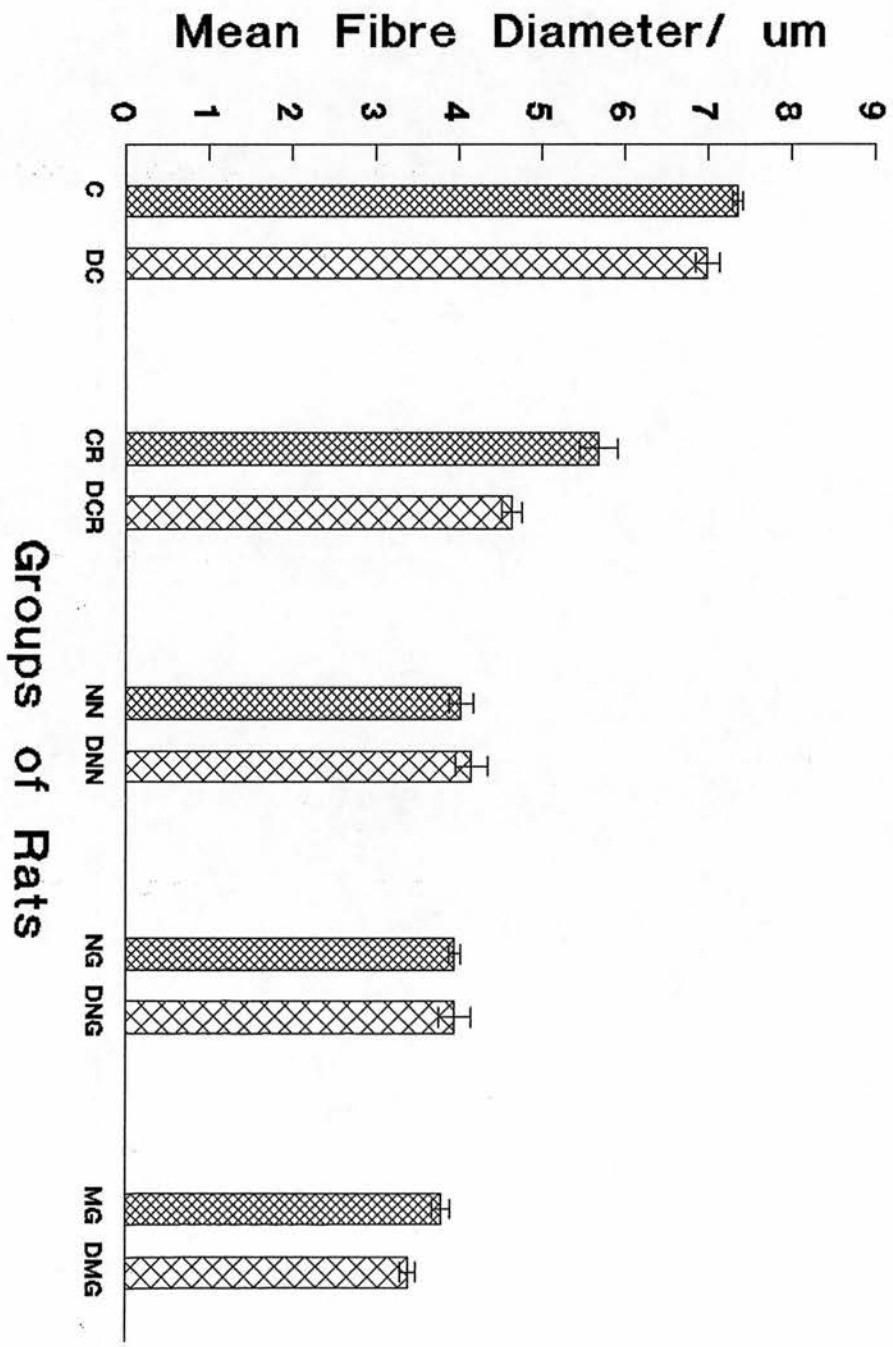
Table 4.21 Table of t values and significance levels for the values of fibre diameter in non-diabetic v diabetic animals (Student's t test)

	C	CR	NN	NG	MG
DC	t=1.56 NS	–	–	–	–
DCR	–	t=4.52 p<0.01	–	–	–
DNN	–	–	t=0.55 NS	–	–
DNG	–	–	–	t=0.01 NS	–
DMG	–	–	–	–	t=1.77 NS

Myelin thickness.

The myelin thickness was calculated by subtracting each axon diameter from each corresponding fibre diameter and then dividing by 2. Approximately 200 values were obtained for each animal, these were averaged to give a value for each rat. The mean, standard deviation, standard error of the mean and coefficient of variation of myelin thickness, for each non-diabetic group is displayed in table 4.22. Table 4.23 shows the equivalent values for the diabetic animal groups. When normal and half-normal plots were made for the values of myelin thickness, 1 outlier was found. The outlier was the value 0.886 μm, belonging to one of the non-diabetic crush animals.

Figure 4.11 Bar chart of the mean fibre diameter of each of the groups of animals (n = 5 for each group)



Ignoring this value, the normal and half-normal graphs were replotted. The data now fitted a normal distribution. The residuals plotted against their expected fitted values showed that each sample had a similar variance. Owing to the fact that 1 value did not fit the normal distribution of the rest of the data, this value was left out of the statistical analysis.

Table 4.22 Values of mean myelin thickness, standard deviation, standard error of the mean and coefficient of variation for non-diabetic animals

RAT GROUP	n	m /μm	SD	SEM	c.v.
C	5	1.64	0.11	0.05	6.5
CR	4	1.55	0.17	0.08	10.85
NN	5	1.04	0.16	0.07	15.71
NG	5	1.10	0.12	0.06	11.25
MG	5	1.10	0.06	0.03	5.55

Table 4.23 Values of mean myelin thickness, standard deviation, standard error of the mean and coefficient of variation for diabetic animals

RAT GROUP	n	m /μm	SD	SEM	c.v.
DC	5	1.56	0.11	0.05	6.99
DCR	5	1.26	0.06	0.03	4.79
DNN	5	1.08	0.13	0.06	11.58
DNG	5	1.14	0.16	0.07	13.77
DMG	5	0.96	0.06	0.03	6.49

Results from significance tests on the values of myelin thickness.

a) F test for variance.

The value of F obtained from the F test on the values of myelin thickness was $F = 17.14$. This result was very significant ($p < 0.001$), indicating that the samples were not from the same populations.

b) Student's t test on the means of myelin thickness.

Non-diabetic rats.

The values of t and the significance levels for the t tests performed on the means of myelin thickness from the non-diabetic animals, are shown in table 4.24. The pattern of significance for myelin thickness was very similar to that for fibre and axon diameter. The only difference was that the mean myelin thickness of the nerve crush group, although smaller than the control group (m = 1.55 μm as compared with m = 1.64 μm, respectively) was not significantly smaller at the 5% level. This is probably because one of the values in the nerve crush group was eliminated as it did not fit the normal distribution of the rest of the data (see above), thus making the t test less sensitive. The nerve-to-nerve suture, nerve graft and muscle graft groups all recovered a significantly smaller thickness of myelin than both the nerve crush and control groups ($p < 0.01$). The nerve-to-nerve suture, nerve graft and muscle graft groups recovered thicknesses of myelin that were not significantly different from each other ($p > 0.05$).

The mean myelin thickness of the nerve graft and muscle graft groups was 1.1 μm in both groups and 1.04 μm in the nerve-to-nerve suture group. Such values imply a similar recovery in each of these groups, however, these results tell nothing of the myelin thickness relative to the axon diameter and hence, maturation of the fibres. It is for this reason that G-ratio was calculated (see below).

Table 4.24 Table of t values and significance levels for the values of myelin thickness in non-diabetic animals (Student's t test)

	C	CR	NN	NG	MG
C	_	t=0.99 NS	t=7.07 p<0.01	t=6.36 p<0.01	t=6.35 p<0.01
CR	_	_	t=5.68 p<0.01	t=5.01 p<0.01	t=5.00 p<0.01
NN	_	_	_	t=0.71 NS	t=0.72 NS
NG	_	_	_	_	t=0.01 NS
MG	_	_	_	_	_

Diabetic rats.

The values of t and the significance levels for the t tests performed on the means of myelin thickness from the diabetic animals, are shown in table 4.25. Comparably to the non-diabetic animals, the mean myelin thickness of the nerve-to-nerve suture, nerve graft and muscle graft groups were very similar in size, m = 1.08, 1.14 and 0.96 μm, respectively. The mean myelin thickness of the nerve crush group did not recover as well as the non-diabetic crush group, in that the mean of the diabetic crush group was significantly smaller than the control mean ($p < 0.05$); this was not the case for the non-diabetic crush and control groups. The nerve-to-nerve suture, nerve graft and muscle graft groups recovered a significantly smaller mean myelin thickness than the control group ($p < 0.01$). The diabetic muscle graft group had a significantly smaller mean myelin thicknes than the nerve crush group ($p < 0.05$). The regenerated fibres of the crush group, however, did not differ significantly in terms of myelin thickness from the other three operated groups ($p > 0.05$). This is probably because of

the poor recovery of myelin thickness in the nerve crush group. None of the other t tests between groups were significant at the 5% level, indicating that the nerve-to-nerve suture, nerve graft and muscle graft groups recovered similarly poorly.

Table 4.25 Table of t values and significance levels for the values of myelin thickness in diabetic animals (Student's t test)

	DC	DCR	DNN	DNG	DMG
DC	–	t=3.58 p<0.05	t=5.71 p<0.01	t=5.03 p<0.01	t=7.12 p<0.01
DCR	–	–	t=2.13 NS	t=1.44 NS	t=3.53 p<0.05
DNN	–	–	–	t=0.68 NS	t=1.41 NS
DNG	–	–	–	–	t=2.09 NS
DMG	–	–	–	–	–

Non-diabetic *v* diabetic rats.

Table 4.26 shows the values of t and the significance levels for t tests performed on the non-diabetic groups and their corresponding diabetic groups. The recovery of myelin thickness was significantly poorer in the diabetic crush group as compared with the non-diabetic crush group ($p < 0.05$). This result indicates that diabetes affects the recovery of myelin thickness in nerve crush injuries. The detrimental effect of diabetes on recovery of myelin thickness in crush injuries was not borne out in the other results. None of the other pairs groups were significantly different in terms of recovery of myelin thickness ($p > 0.05$).

Table 4.26 Table of t values and significance levels for the values of myelin thickness in non-diabetic v diabetic animals (Student's t test)

	C	CR	NN	NG	MG
DC	t=0.90 NS	–	–	–	–
DCR	–	t=3.23 p<0.05	–	–	–
DNN	–	–	t=0.47 NS	–	–
DNG	–	–	–	t=0.44 NS	–
DMG	–	–	–	–	t=1.66 NS

G-ratio.

The mean, standard deviation, standard error of the mean and coefficient of variation of G-ratio for each group of non-diabetic rats is diaplayed in table 4.27 and the equivalent for the diabetic groups of rats is displayed in table 4.28. Normal and half-normal plots were made for the values of G-ratio. 2 outliers were found, one belonging to the non-diabetic crush group (G-ratio = 0.605) and the other belonging to the the non-diabetic muscle graft group (G-ratio = 0.521). Normal and half-normal plots were made excluding these 2 outliers. Straight lines were obtained, showing that the rest of the data were normally distributed. The residuals plotted against the fitted values of the mean for each group, showed that each sample had a similar variance. Owing to the fact that 2 of the values did not fit the normal distribution of the rest of the data, they were not included in the statistical tests.

Table 4.27 Values of the mean, standard deviation, standard error of the mean and coefficient of variation of G-ratio in non-diabetic animals

RAT GROUP	n	m	SD	SEM	c.v.
C	5	0.54	0.03	0.01	5.61
CR	4	0.46	0.03	0.01	6.05
NN	5	0.46	0.05	0.02	10.90
NG	5	0.43	0.04	0.02	10.22
MG	4	0.38	0.01	0.01	3.27

Table 4.28 Values of the mean, standard deviation, standard error of the mean and coefficient of variation of G-ratio in diabetic animals

RAT GROUP	n	m	SD	SEM	c.v.
DC	5	0.54	0.02	0.01	4.41
DCR	5	0.43	0.05	0.02	10.47
DNN	5	0.46	0.06	0.03	12.79
DNG	5	0.41	0.05	0.02	11.47
DMG	5	0.41	0.04	0.02	9.30

Results from significance tests on the values of G-ratio.

a) F test for variance.

The value of F obtained from the F test on the data of G-ratio was $F = 6.77$, d.f. = 9, 47. This result was significant ($p < 0.001$) which indicated that the samples were not from the same population.

b) Student's t tests on the means of G-ratio.

Non-diabetic rats.

The t values and significance levels of t tests performed on the values of G-ratio from non-diabetic animals, are displayed in table 4.29. The mean G-ratio of the nerve crush and nerve-to-nerve suture groups was smaller than the mean of the control group; $m = 0.46$ for both crush and

nerve-to-nerve suture groups as compared with m = 0.54 for the control group. However, the group means were not significantly different from each other at the 5% level. The mean of the nerve graft group , m = 0.43, was significantly smaller than the control group ($p < 0.01$), as was the mean of the muscle graft group, m = 0.38, ($p < 0.01$). None of the other group means was significantly different from any other ($p > 0.05$).

Table 4.29 Table of t values and significance levels for the values of G-ratio in non-diabetic animals (Student's t test)

	C	CR	NN	NG	MG
C	_	t=2.54 NS	t=2.65 NS	t=3.92 p<0.01	t=5.21 p<0.01
CR	_	_	t=0.05 NS	t=1.15 NS	t=2.53 NS
NN	_	_	_	t=1.27 NS	t=2.71 NS
NG	_	_	_	_	t=1.51 NS
MG	_	_	_	_	_

G-ratio is a measure of myelinated fibre maturation. The mean G-ratio of each group indicates that the more severe the nerve injury is, the poorer the maturation of myelinated fibres. Only the groups with the most severe injuries recovered a significantly smaller G-ratio i.e. nerve and muscle graft.

Diabetic rats.

The t values and significance levels of t tests performed on the values of G-ratio from diabetic animals, are displayed in table 4.30. The mean G-ratio was smallest for the most severe injuries; m = 0.41 for both the nerve and muscle graft groups. In both cases, the mean was significantly

smaller than the control mean, m = 0.54, ($p < 0.01$). The mean of the nerve crush group, m = 0.43, was significantly smaller than the control mean ($p < 0.01$). The mean G-ratio of the nerve-to-nerve suture group, m = 0.46, was bigger than the crush group and not significantly smaller than the control group ($p > 0.05$). None of the other pairs of means were significantly different from each other ($p > 0.05$).

Table 4.30 Table of t values and significance levels for the values of G-ratio in diabetic animals (Student's t test)

	DC	DCR	DNN	DNG	DMG
DC	–	t=3.69 p<0.05	t=2.74 NS	t=4.65 p<0.01	t=4.32 p<0.05
DCR	–	–	t=0.95 NS	t=0.97 NS	t=0.64 NS
DNN	–	–	–	t=1.96 NS	t=1.59 NS
DNG	–	–	–	–	t=0.33 NS
DMG	–	–	–	–	–

The most severe of the injuries showed poorest fibre maturation i.e. the nerve and muscle grafted animals. Surprisingly, the nerve crush animals recovered less well than the nerve-to-nerve suture group.

Non-diabetic *v* diabetic rats.

Table 4.31 shows the t values and significance levels of tests performed on the corresponding pairs of group means, from the diabetic and non-diabetic populations. None of the 5 tests were significant at the 5% level. This demonstrates that the degree of maturation of nerve fibres in the diabetic rats, after nerve injury and repair, was comparable to the maturation of nerve fibres in non-diabetic rats after a similar injury.

Table 4.31 Table of t values and significance levels for the values of G-ratio in non-diabetic v diabetic animals (Student's t test)

	C	CR	NN	NG	MG
DC	t=0.02 NS	–	–	–	–
DCR	–	t=0.95 NS	–	–	–
DNN	–	–	t=0.11 NS	–	–
DNG	–	–	–	t=0.75 NS	–
DMG	–	–	–	–	t=1.12 NS

Plate 4.1 Photograph of a transverse section of the peroneal nerve, stained with toluidine blue, from a rat in the non-diabetic control group (X40)

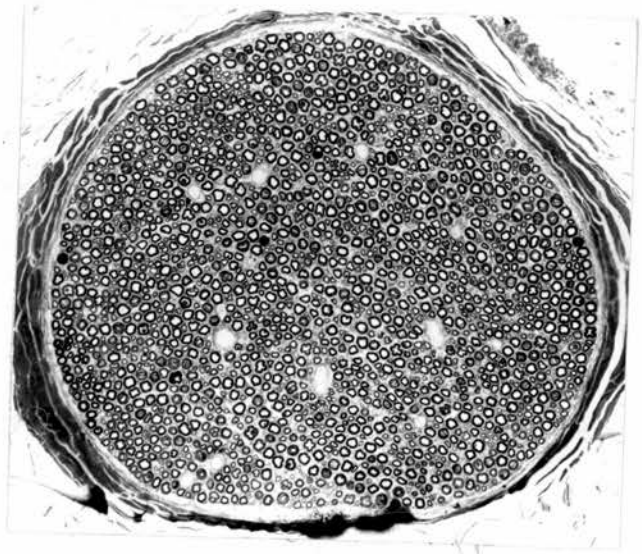


Plate 4.2 Photograph of a transverse section of the peroneal nerve, stained with toluidine blue, from a rat in the diabetic control group (X40)

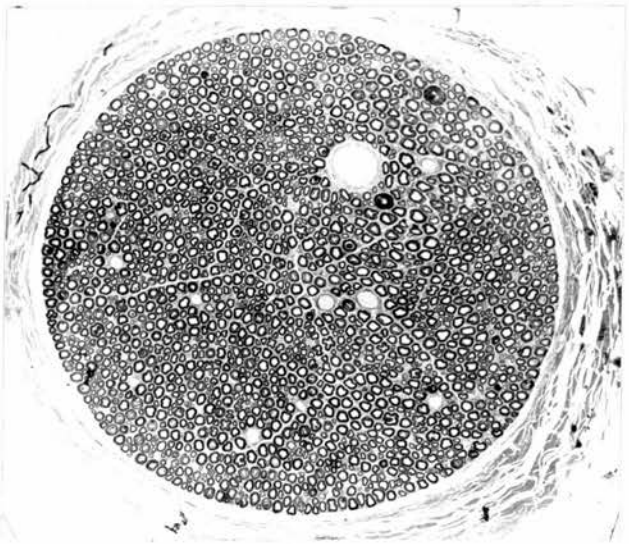


Plate 4.3 Photograph of a transverse section of the peroneal nerve, stained with toluidine blue, from a rat in the non-diabetic crush group (X40)

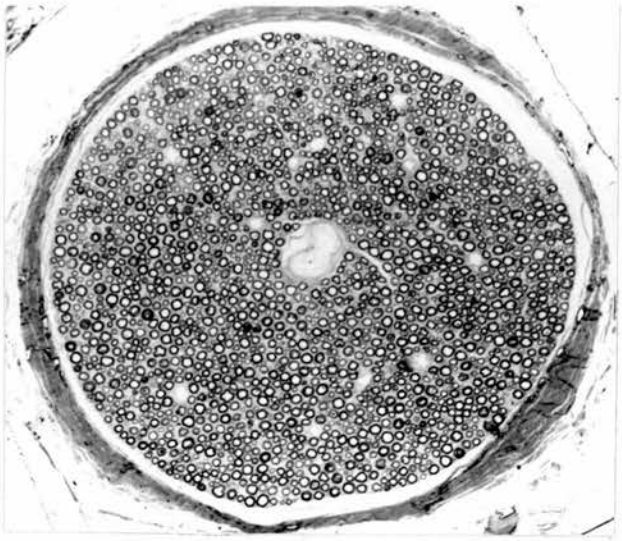


Plate 4.4 Photograph of a transverse section of the peroneal nerve, stained with toluidine blue, from a rat in the diabetic crush group (X40)

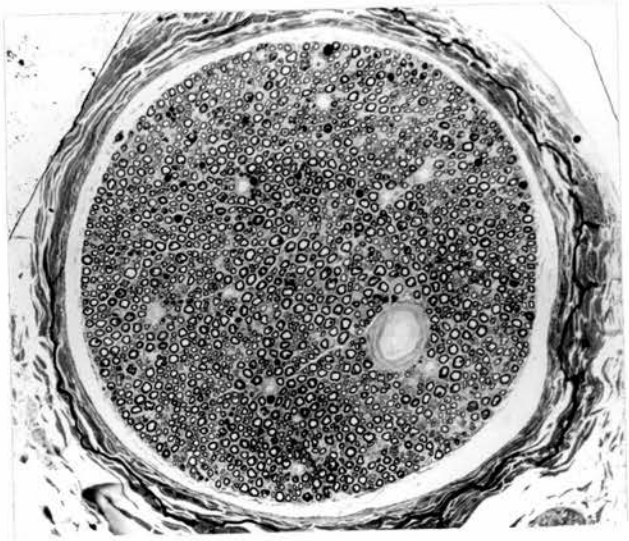


Plate 4.5 Photograph of a transverse section of the peroneal nerve, stained with toluidine blue, from a rat in the non-diabetic nerve-to-nerve suture group (X40)

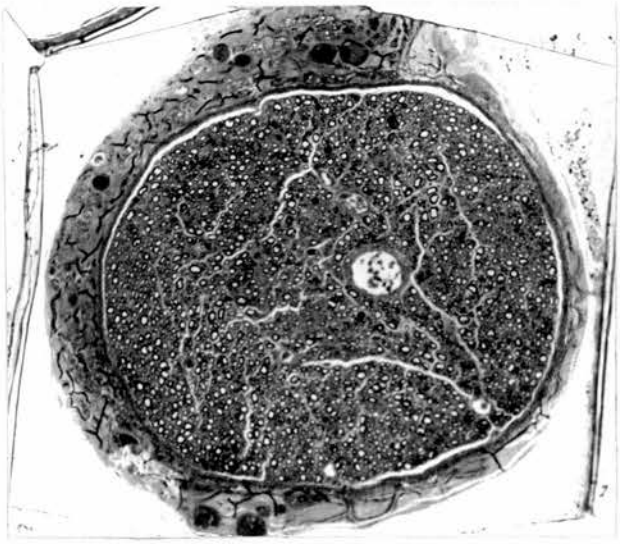


Plate 4.6 Photograph of a transverse section of the peroneal nerve, stained with toluidine blue, from a rat in the diabetic nerve-to-nerve suture group (X40)

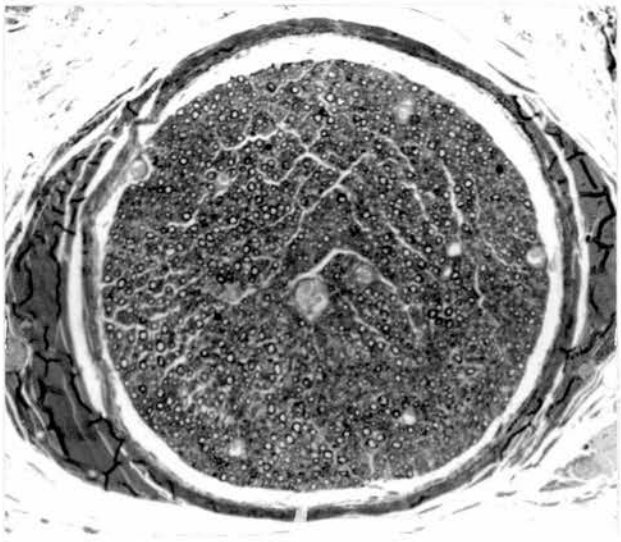


Plate 4.7 Photograph of a transverse section of the peroneal nerve, stained with toluidine blue, from a rat in the non-diabetic nerve graft group (X40)

Plate 4.8 Photograph of a transverse section of the peroneal nerve, stained with toluidine blue, from a rat in the diabetic nerve graft group (X40)

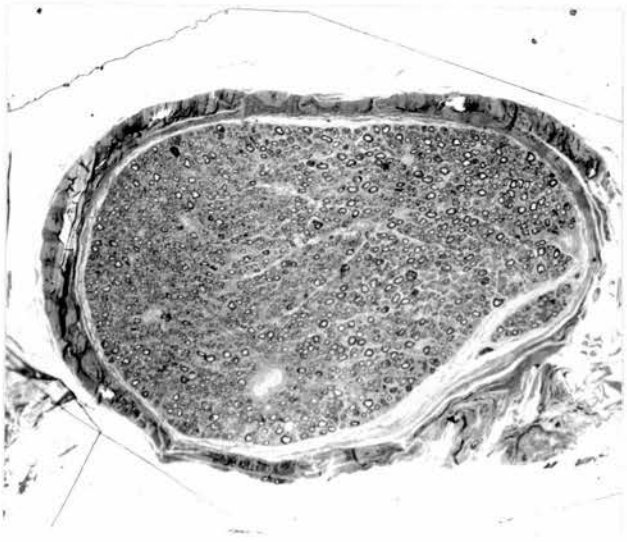


Plate 4.9 Photograph of a transverse section of the peroneal nerve, stained with toluidine blue, from a rat in the non-diabetic muscle graft group (X40)

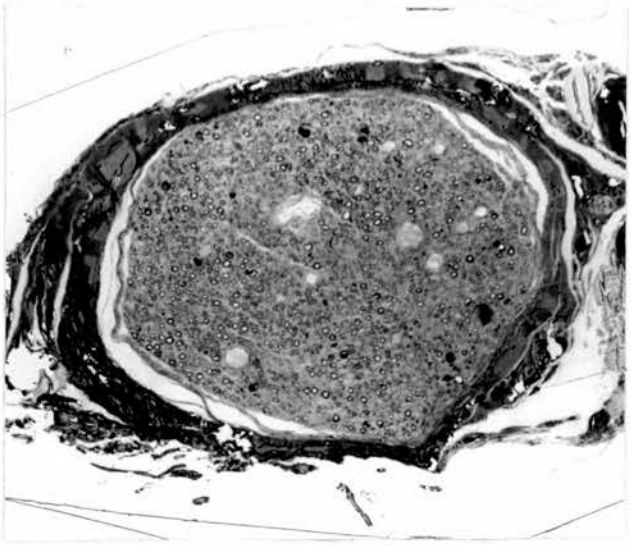
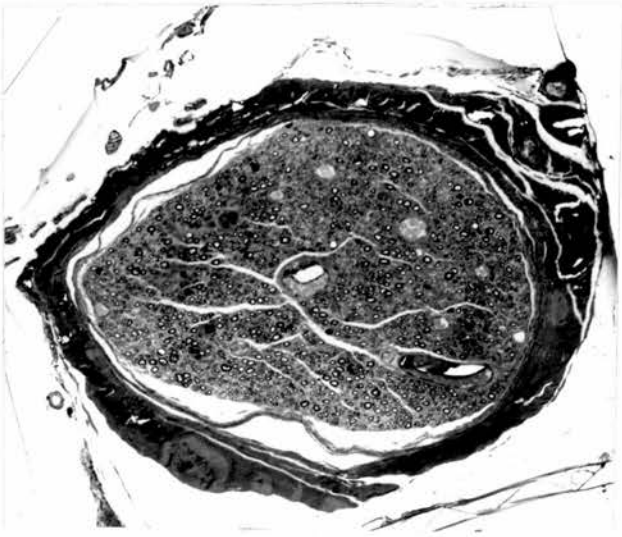


Plate 4.10 Photograph of a transverse section of the peroneal nerve, stained with toluidine blue, from a rat in the diabetic muscle graft group (X40)



4.4 Discussion.

4.4.1 Conduction Velocity in Non-diabetic Rats.

When measuring conduction velocity after nerve injury and regeneration, the results depend on where the measurements are made, whether it be proximal or distal to the site of the injury (Berry, Grundfest and Hinsey, 1944). All of the measurements carried out in the experiments of this thesis, were made over 1.5-2 cm lengths of sciatic nerve, and the site of injury was mid-point in this length. It was possible to detect the site of injury in the nerve-to-nerve sutured animals, nerve grafted and muscle grafted animals because of the suture lines. In the case of the nerve crush groups, the crush had been produced at a consistent site in all animals (chapter 2.6) and this site was used as the mid-point for recording conduction velocity.

In the work presented in this thesis, the average conduction velocity recorded from the fastest conducting fibres of the left sciatic nerves of the non-diabetic control group was 44.82 ms^{-1}. This was very similar to the average conduction velocity recorded from the right (unoperated) sciatic nerves of the 51 non-diabetic rats; which was 44.79 m s^{-1}. These values are smaller than those recorded by other researchers working in the same laboratory, Myles and Glasby (1991), who recorded an average conduction velocity of 70 m s^{-1} from the sciatic nerves of rats. However, it should be noted that these authors compiled data from the unoperated sciatic nerves of animals, 50-300 days after operating on the contralateral sciatic nerve. In the experiments presented here, the mean conduction velocity of the left sides of the non-diabetic control group was very similar to the mean

conduction velocity of the unoperated sides of the non-diabetic animals and therefore, the fact that Myles and Glasby (1991) did not have a separate control group is probably not a significant failing. However, the mean conduction velocity value of 70 m s^{-1} was obtained from 48 rats which varied in age by up to 250 days. In the rat, conduction velocity is known to increase up to 9 months of age (Sharma & Thomas, 1974) and considering the fact that the rats of Myles and Glasby (1991) were 'immature' at the time of operating, it would seem likely that the nerves of the animals left for 300 days to recover would inevitably conduct faster than the rats left for only 50 days. In truth, these rats should not have been included in the same control group. Also, the statistics performed on the data were used to compare groups whose sample sizes varied between n = 4 and n = 48. Birren and Wall (1956) demonstrated that the conduction velocity of rat sciatic nerves varied from 26 ms^{-1} at 60 days to 53 ms^{-1} at 350 days. The animals used in the work of this thesis were somewhat younger than the 350 day rats of Birren and Wall and would, therefore, be expected to have lower conduction velocities than 53 m s^{-1}. It would seem that the mean conduction velocity of the non-diabetic control nerves obtained in the work presented here, is much more in keeping with the results of Birren and Wall (1956) than that of Myles and Glasby (1991).

4.4.2 Conduction Velocity in Diabetic Rats.

In chapter 3.3.1, it was noted that the average body weight of the diabetic animals was lower than that of the non-diabetic animals. It is possible that differences in body weight could affect values of conduction velocity, however, Birren and Wall (1956) stated that conduction velocity does not appear to be related to body weight in adult rats. Also, one of the advantages of expressing the conduction velocity of the operated side as a

fraction of the unoperated side, as used in the work presented here, is that age and maturation differences from animal to animal are eliminated.

The proposed reasons for the reduction of conduction velocity in diabetic rats are discussed in section 4.1. Eliasson (1964) demonstrated a reduction in nerve conduction velocity by 26% and 28% in sensory and motor fibres of the tibial nerve, respectively, 14 days after administration of alloxan. This, he found, correlated with the reduction of conduction velocity of the whole nerve, from 52 ± 3 ms^{-1} to 41 ± 2 ms^{-1}. Greene et al.(1975) found a similar reduction of approximately 27% of motor nerve conduction velocity in STZ rats, 14 days after administration of STZ. In the work presented here, the reduction of whole nerve conduction velocity was 39%. Such a large reduction in nerve conduction velocity could be partly due to the fact that the recorded values were expressed as a percentage of the small mean conduction velocity of the diabetic control group. The mean value of conduction velocity obtained from the diabetic control group was 27.26 ms^{-1}, however, the standard deviation was unusually large: 8.36. The greater reduction in conduction velocity seen in the diabetic controls of the work presented here compared with that of the authors above, could be because the rats used in this work had been diabetic for over 150 days as compared to the 14 days of the rats used by Eliasson (1964) and Greene *et al.* (1975). Jakobsen (1979) reported a progressive decrease in conduction velocity with time in his STZ diabetic rats.

From appendix 7 (which shows the values of conduction velocity recorded from operated and unoperated sciatic nerves in both non-diabetic and diabetic animals), it can be seen that the range of values of conduction velocity obtained from the unoperated sides of the diabetic animals varied enormously from rat to rat compared to the non-diabetic animals. This variation may have been due to a range in the severity of the diabetes (see

chapter 3). The coefficient of variation of conduction velocity in the diabetic control group was 30.68% (table 4.2). This figure is the standard deviation expressed as a percentage of the mean and is an indication of the variation in conduction velocity between animals within one group. However, this variation was not large when measurements were made from individual animals. From appendix 7 it can be seen that the conduction velocities of left and right sciatic nerves recorded from each animal in the diabetic control group were very similar. This is demonstrated by the fact that the coefficient of variation of the CV ratio for the diabetic control group (table 4.4) was only 12.97%. The variation in conduction velocity between diabetic animals is another reason why the CV ratio was a better value to use in statistical tests (chapter 4.3.2).

In their experiments, Thomas, Jefferys, Sharma and Bajada (1981) demonstrated a reduction of only 2.2 m s^{-1} of motor nerve conduction velocity in STZ diabetic rats. They argued that this small reduction in conduction velocity was probably because their rats were mature (over 9 months of age) before the administration of STZ. Consequently, the motor nerve conduction velocities of their rats would not be expected to increase any further as a result of maturation and, therefore, the limited reduction in conduction velocity that they observed was the true effect of diabetes. They proposed that the large reductions in conduction velocity observed by other authors were due to a failure of nerve fibres to mature in STZ diabetic rats. If that which Thomas *et al.* (1981) claimed is correct, then the modest decrease in conduction velocity caused by the diabetic state was hardly enough for other authors to use as an indication of 'neuropathy', especially when no other morphological signs are present (see below), and to extrapolate results and treatments from experimental diabetes to the human situation would be inappropriate. However, a decreased conduction velocity

continues to be the subject of investigations and is still considered by many to be one of the prime indicators of neuropathy in experimental diabetes.

4.4.3 Recovery of Conduction Velocity.

The work of Cragg and Thomas (1964) on the recovery of conduction velocity after nerve crush injuries of the peroneal nerve in rabbits, showed that conduction velocity returned to approximately 75% of control values when measured at 12-16 months. In the work presented here, recovery of conduction velocity was 74.59% in the diabetic crush group and 72.7% in the non-diabetic crush group. These values, although obtained after only 150 days, are very similar to the findings of Cragg and Thomas (1964) and suggest that maximium recovery of conduction velocity after a crush injury may well have been achieved as early as 150 days after the injury had occurred in the rat.

Difficulties arise when comparing crush injuries because the severity of the crush can vary from one group of researchers to another. The traditional method of producing a crush was to apply a watchmaker's forceps to a nerve for approximately 10 seconds (Cragg & Thomas, 1961; Sharma & Thomas, 1975; Ekström & Tomlinson, 1989). Obviously the pressure put on the nerve can vary enormously from animal to animal. In the work of this thesis, a more reproducible crush was made using smooth tipped artery forceps (chapter 2.5). The fact that the recovery of conduction velocity was poorer after nerve crush than after nerve-to-nerve suture, in both diabetic and non-diabetic animals, suggests that a more severe injury than the intentional axonotmesis (or Sunderland second degree injury) could have been produced as a result of the crush. In the Sunderland third degree injury, the perineurium remains intact but there is scarring in the endoneurium resulting in prevention of some regenerating fibres from

reaching end-organs. In this type of injury, the variation of recovery is broadest, from almost complete recovery to almost no recovery (Mackinnon & Dellon, 1988). However, the standard deviation of conduction velocity (see tables 4.1 and 4.2) for the crush groups was no larger than for the other repair groups, showing that the recovery of conduction velocity after nerve crush injury was quite consistent from animal to animal.

Berry, Grundfest and Hinsey (1944) showed from their work on the peroneal and tibial nerves in cats that nerve conduction velocity never returned to normal after nerve-to-nerve suture. They found that the recovery of conduction velocity returned to approximately 80% of control values by 466 days. The findings from the nerve-to-nerve suture groups in the work presented here show similar returns of conduction velocity to those of Berry *et al.* (1944). In the work presented here, the return of conduction velocity to 83.23% and 79.92% of control values were obtained from non-diabetic and diabetic rats, respectively, 150 days after nerve-to-nerve suture. Myles and Glasby (1991) demonstrated a return of conduction velocity to only 58% of controls 300 days after nerve-to-nerve suture. The reason why the results of these authors were so much poorer than those of Birren *et al.* (1944) and those presented in this thesis could possibly be because, in operating on the sciatic nerve, the nerve stumps had not been freed completely, resulting in tension at the suture line. However, it is more likely that a better explanation for the poor results obtained by Myles and Glasby (1991) after nerve-to-nerve suture is that these authors were expressing the conduction velocity after repair as a percentage of an abnormally high control value (see above).

After nerve grafting, the recovery of conduction velocity in the experiments of this thesis was 48.33% and 52.00% of the controls in the non-diabetic and diabetic groups, respectively. A poorer recovery than that

after direct epineurial suture is to be expected as the regenerating fibres had to overcome two suture lines and the inevitable scarring that is associated with this. The recovery of conduction velocity after muscle grafting was poorer than after nerve grafting; with returns of only 28.41% and 26.15% of control values in the non-diabetic and diabetic animals, respectively. These results are not dissimilar to those of Myles and Glasby (1991) who demonstrated returns of conduction velocity to 36% of controls after 300 days. These authors also showed that improvements in conduction velocity occurred after 150 days in nerves that had been repaired by nerve-to-nerve suture and muscle grafting. It is, therefore, to be expected that had the rats used in the work described in this thesis been left for a longer time to recover, the conduction velocities recorded would have been higher. The recovery of CV ratio in the muscle graft groups was not significantly poorer than the recovery of CV ratio in the nerve graft groups, in both diabetic and non-diabetic animals.

The degree of recovery of conduction velocity of the diabetic animals was very similar to the non-diabetic animals and no difference was found in statistical tests. The results obtained from this work indicate success in producing a standardized crush procedure that gives results that are both reproducible and consistent, although, the injury produced may be closer to a Sunderland third degree injury than a second degree injury. A pure type II injury could most likely be arrived at by varying the time of the crush and the extent to which the ratchet was closed (see chapter 2.5 in which the crush procedure used in the work presented here is described). This could be ascertained experimentally by comparing damage induced in the nerve histologically and electrophysiologically. Direct nerve-to-nerve epineurial suture produced the best recovery in terms of conduction velocity. Nerve grafting produced poorer recovery in terms of conduction velocity than

nerve-to-nerve suture, although the poorest return was seen in the muscle grafted animals. However, the recovery of conduction velocity was not significantly poorer in the muscle graft groups as compared to the nerve graft groups. Very similar recovery of nerve conduction velocity was obtained in the diabetic animal groups. Although absolute conduction velocity values were slower in the diabetic animals than in non-diabetic animals, the diabetic state did not make any difference to the degree of recovery of conduction velocity after nerve injury and repair.

4.4.4 Recovery of Nerve Morphology.

In section 4.1.2 the relationship between nerve conduction velocity and nerve morphology is discussed. From the results on conduction velocity it was expected that the poorest recovery of nerve fibre and axon diameter would be found in the nerve and muscle graft groups; this was found to be true and is discussed below. Of particular interest was the recovery of fibre and axon diameter in the diabetic animals. It is commonly believed that the effect of diabetes is primarily an effect on the Schwann cell (Wattig *et al.*, 1991) and, therefore, the degree of maturation (G-ratio) recovered after nerve injury and repair was of great interest.

In the work presented here, the recovery of mean fibre diameter, 150 days after nerve crush in the non-diabetic animals, when expressed as a percentage of mean fibre diameter of the non-diabetic control group, was 77.14%. The equivalent percentage recovery in the diabetic animals was 66.24%. Although the work of Gutmann and Sanders (1943) was performed on the peroneal nerves of rabbits and, therefore, cannot be used as an absolute comparison to the work of this thesis, their results can be used as an indication of the type of recovery to be expected after different types of nerve injury and repair. Gutmann and Sanders (1943) showed the average

fibre diameter, 130 days after crushing the peroneal nerve in rabbits, to range from 3.64 to 3.93 μm and the control values of average fibre diameter ranged from 5.26 to 5.56 μm. When the value of average fibre diameter was expressed as a percentage of their control values, they obtained a percentage recovery somewhere in the range of 65.47% to 74.57%, very similar, in fact, to the findings of the experiments presented in this thesis. By 300 days the average fibre diameter measured by Gutmann and Sanders reached control values. These authors also performed nerve-to-nerve suture experiments on their rabbits and found very variable results. They recorded average fibre diameters from 3.16 to 3.61 μm after 200 days recovery time. If expressed as a percentage of their control values, the recovery after nerve-to-nerve suture was in the range of 56.83% to 68.5%. The percentage recovery of mean fibre diameter after nerve-to-nerve suture in the experiments described here was 54.67% in the non-diabetic animals and 59.23% in the diabetic animals. These recovery values are slightly smaller than those of Gutmann and Sanders but the values were obtained after only 150 days as compared to the 200 days of the aforementioned authors. Gutmann and Sanders found similar recovery rates after nerve grafting and only after nerve crush was the peroneal nerve fully reconstituted by 364 days. In the experiments presented here, the recovery of fibre diameter 150 days after nerve grafting was 53.61% of control values in non-diabetic animals and 56.37% in the diabetic animals. After muscle grafting, the percentage recovery of fibre diameter in the non-diabetic animals was 51.43% and 48.35% in the diabetic animals. Similarly to the findings of Myles and Glasby (1991), the recovery of fibre diameter in the muscle graft groups were not significantly different from the recovery of fibre diameter in the nerve graft groups, whether the rats were diabetic or not ($p > 0.05$). Also in agreement with these authors, the values of mean fibre

diameter in the nerve-to-nerve suture and muscle graft groups were significantly smaller than control values ($p < 0.01$); and the mean fibre diameter of the muscle graft group was significantly smaller than the mean fibre diameter of the nerve-to-nerve suture group ($p < 0.05$). When comparing the means of fibre and axon diameters from like diabetic and non-diabetic groups, the results were very interesting. The mean fibre diameter of the diabetic control group was 6.99 μm which was smaller than the mean of the non-diabetic control group (7.35 μm), although they were not significantly different ($p > 0.05$). Similar differences were obtained from the measurements of axon diameter. The mean of the diabetic control group was 3.86 μm as compared with a mean of 4.07 μm obtained from the non-diabetic control group. Sigimura, Windebank, Natarajan, Lambert, Schmid and Dyck (1980) reported that although axon circumference and myelin lamellae were unchanged in STZ diabetic nerve, the axonal area was less. They suggested that this was possibly because of an elevated extra cellular osmolarity which resulted in axonal shrinkage during processing of diabetic nerve. If this is accepted as a true effect then the slightly smaller values of fibre and axon diameter measured in the diabetic animals is to be expected and is not to do with a decrease in diameter because of the diabetic state. However, this hypothesis cannot be confirmed because measurements of osmolarity were not made.

Another possible explanation for the smaller fibre and axon diameters measured in the diabetic rats is the retardation of growth of both fibre diameter and skeletal growth observed in STZ diabetic rats (Thomas, Jefferys, Sharma and Bajada, 1981). Should the normal increase in fibre diameter with age (Birren & Wall, 1956, Cragg & Thomas, 1964) fail to occur in STZ diabetic rats , this would explain the findings of the work presented here, although not statistically significant, of smaller fibre and axon

diameters in the diabetic control groups. Further experiments comparing nerve fibre and axon diameters, between STZ diabetic and non-diabetic rats, immediately (perhaps 1 week) after induction of the diabetic state could be performed which would determine whether the smaller diameters measured in the diabetic rats 150 days after operating were mere shrinkage artefact or were due to retardation of fibre growth in STZ diabetic animals. The smaller measurements of fibre and axon diameter in the diabetic operated groups could also be due to shrinkage artefact. Again, these were not significantly different from their non-diabetic equivalents, except the crush groups (see below). However, smaller fibre and axon diameters in diabetic rats as compared to non-diabetics would not be expected, if the recovery rate of diabetic and non-diabetic nerve were the same, and the reason for the smaller control values of fibre and axon diameters seen in diabetics were because of a retardation in growth. This is because after operating, the nerve fibres in both diabetic and non-diabetic animals regenerated from their proximal stumps to their current size in 150 days and were probably not subject to age maturational differences. Sharma, Thomas and De Molina (1977) found the average fibre diameter of the tibial nerve in diabetic rats to be $6.21 \pm 0.52\ \mu m$ and $6.56 \pm 0.32\ \mu m$ in non-diabetic rats. Although the values of fibre diameter measured by Sharma *et al.* (1977) were slightly smaller than the values of fibre diameter of the control groups in the work presented here, the experiments of these authors were of only 5 weeks duration and their animals were almost certainly far younger than those used in the work of this thesis. Sharma *et al.* (1977) demonstrated a significant increase in maximum fibre diameter in their non-diabetic control rats over the 5 week period of their experiment and it is, therefore, to be expected that the nerve fibres measured in the experiments of this thesis would have larger diameters considering the likelihood of the animals being

older than those of the aforementioned authors. Also similar to the findings of the work presented in this thesis, these authors failed to show a significant reduction in maximum or average fibre diameter as a consequence of STZ diabetes although the average fibre diameter of the diabetic rats was smaller than the non-diabetics.

Surprisingly, the mean fibre and axon diameters measured in the diabetic crush group were significantly smaller than those measured in the non-diabetic crush group. If this were simply the result of axonal shrinkage (Sigimura *et al.*, 1980), it would be expected that the control groups in the diabetic and non-diabetic states would also be significantly different from each other. As this was not the case, it can only be supposed that the significance of the difference between the crush groups was due to a poorer recovery of the diabetic animals after nerve crush. Sharma and Thomas (1975) did not find any difference in total fibre count, size-frequency distribution or average diameter at 18 weeks after a crush injury to the sural nerve in STZ diabetic rats. However, the sample size of animals left to recover for 18 weeks was $n = 1$ and the authors did not explain their criteria for induction of the diabetic state, nor how the blood sugar levels were analysed or maintained. It is, therefore, a possibility that the rats used by Sharma and Thomas were not as 'diabetic' as those used in the work presented here. Differences in the rate of regeneration in STZ diabetic rats have been observed by various authors (Ekström & Tomlinson, 1989; Ekström *et al.*, 1989; Triban *et al.*, 1989; Longo *et al.*, 1986), however, most of the work that has been performed on regeneration rates in diabetes has been done on short-term regeneration i.e. 1 to 4 weeks after nerve injury. Ekström and Tomlinson (1989) demonstrated the rate of recovery in diabetic rats to be $15.8 \pm 1.8\,\mu m$ in 7 days as compared to $21.8 \pm 1.2\,\mu m$ in non-diabetic animals. By the 150 days recovery that was allowed in the

experiments described here, it would be expected that regenerating fibres in both non-diabetic and diabetic rats would have reached end organs. The difference in fibre and axon diameter observed after the nerve crush experiments presented in this thesis is not necessarily indicative of a poorer regeneration rate in nerves of STZ diabetic rats but is an indication of a delay in recovery of nerve fibres to full size. In fact, Longo *et al.* (1986) noted the immaturity of axons, 4 weeks after a crush injury in STZ diabetic rats and suggested that regeneration in such animals is not only "significantly delayed" but also "qualitatively impaired".

Noting the impairment of recovery of axon and fibre diameter after nerve crush in diabetic rats as compared to the non-diabetic animals, it would be expected that the recovery of axon and fibre diameter after nerve-to-nerve suture, nerve graft and muscle graft would also be poorer in the diabetic groups than their equivalent non-diabetic groups. However, no significant difference was found, although the mean fibre and axon diameters of the diabetic nerve and muscle graft groups were slightly smaller than their non-diabetic equivalents. Although the slightly smaller diameters measured in the diabetics might be explained by axonal shrinkage (see above), another possible explanation for the significance of the crush groups is that after repairing the nerve by either nerve-to-nerve suture, nerve graft or muscle graft the degree of recovery, in terms of axon and fibre diameter, is so poor in the non-diabetics that a significant difference between these rats and their diabetic equivalents is not found. With injuries of the neurotmesis type, regeneration through suture lines and the establishment of appropriate pathways are more dominant problems than any superimposing metabolic disorder.

As discussed above, it is possible that a Sunderland type III injury had been produced in the crush groups. Recovery after a type III injury can vary

enormously and it is possible that the recovery of the diabetic crush group was particularly poor or the recovery of the non-diabetic crush group particularly good, thus resulting in significantly smaller fibre and axon diameters in the diabetic crush group than the non-diabetic crush group. Should a Sunderland type III injury have been induced, it would be more likely that a wide range of recovery of fibre and axon diameter had been obtained. However, the coefficient of variation for axon and fibre diameters were not dissimilar to the other operated groups in both non-diabetic and diabetic animals, and were, in fact, smaller in 3 out of the 4 situations (tables 4.12, 4.13, 4.17, 4.18). Importantly, although the measurements of fibre diameter, axon diameter and G-ratio from the diabetic control group were not dissimilar to their non-diabetic equivalents, the conduction velocities recorded from the diabetic animals were much smaller than those in the non-diabetics. After nerve injury and repair, the diabetic animals showed similar reductions in conduction velocity to the non-diabetic animals and, with the exception of the diabetic crush group, recovery of fibre diameter, axon diameter and G-ratio were very similar in non-diabetics as compared to the diabetic animals. This would substantiate the belief that it is the structural changes that occur as a result of nerve injury and regeneration that lead to a decrease in nerve conduction velocity, however, the basis of the decrease in conduction velocity in diabetic animals is a biochemical one.

In conclusion, the work described in this chapter has confirmed that the diabetic state in rats causes a reduction in nerve conduction velocity which is unlikely to be caused simply by a failure in the maturation of nerve fibres. The recovery of conduction velocity was best after nerve-to-nerve suture and after nerve crush, and poorest after muscle and nerve grafting, although it is likely that complete recovery had not occurred in the latter

groups. The recovery of conduction velocity was not significantly poorer in the diabetic animals.

By 150 days, the axon and fibre diameters had not returned to normal in any of the operated groups and the recovery of fibre diameter was worse than the recovery of axon diameter. The recovery was the best in the crush groups and worst in the muscle graft groups, although the recovery after muscle grafting was not significantly poorer than after nerve grafting. The fibre and axon diameters were smaller in the diabetic control group as compared to the non-diabetic control group, although not significantly so ($p > 0.05$), however, the recovery of axon and fibre diameters were significantly poorer in the diabetic crush group as compared to the non-diabetic crush group ($p < 0.01$). It is considered that the most likely explanation for this difference and the lack of a difference in the recovery of axon and fibre diameters in the other operated groups, is that recovery after nerve injury and repair is, in fact, poorer in diabetic rats. The recovery of fibre and axon diameters were also poor in the non-diabetic nerve-to-nerve suture, nerve graft and muscle graft groups and consequently, the difference in recovery between like groups of diabetic and non-diabetic animals was too small to show up in significance tests for groups of such small numbers.

It has been discussed in the introduction to this thesis that results from studies on the recovery of conduction velocity, fibre size and myelination do not provide information about the degree of functional recovery that can be expected after nerve injury and repair. This is because restoration of function occurs before fibres have matured completely after nerve regenerataion (Gutmann *et al.*, 1942) and also because recovery of fibre diameter *etc.* gives no indication about how many fibres have reached appropriate targets after reinnervation. In the work described in the

following 3 chapters, the degree of functional recovery after nerve injury and repair was investigated.

CHAPTER 5

Assessment of Recovery of Cutaneous Receptive Fields

5.1 Introduction.

5.1.1 Aims.

Almquist and Eeg-Olofsson (1970) investigated conduction velocity of sensory nerves and two-point discrimination in humans after the repair of injured nerves by nerve-to-nerve suture. They noticed that sensory nerve conduction velocity, which normally correlates well with fibre diameter, does not correlate with clinical results. They said that when assessing functional recovery: "Velocity change ,... is not a crucial factor". Naturally, a patient is more likely to be concerned about the recovery of sensation after injury of a sensory nerve than the velocity of impulse conduction of the same. Therefore, this and the subsequent two chapters were designed to investigate recovery of function after nerve injury and repair.

The aims of this chapter are to investigate the recovery of the peroneal cutaneous receptive field after different types of nerve injury and repair (see chapter 2.5), and to compare the recovery, after 150 days, with similar repair groups of diabetic animals.

5.1.2 Methods of Assessing the Recovery of Sensory Function.

There are different methods by which it is possible to assess the recovery of sensation after nerve injury and repair.

(a) Psycho-physiological studies. Such studies include tests of two-point discrimination which are performed on conscious patients (Almquist & Eeg-Olofsson, 1970). The advantage of this method is that results can be obtained directly from humans and no extrapolation of results

from animal experimentation is necessary. The limitation of this method is that the results are somewhat subjective because they rely on the patients' interpretation of the sensory stimuli.

(b) Histological studies. These studies can be performed on both humans and animals, however, the results are disadvantaged by the fact that this method measures structure not function and thus, can only ever give an indication of function of, or within, cells.

(c) Electrophysiological studies. These methods involve the recording of afferent impulses from either whole nerve or single fibres. For ethical reasons, experiments such as these are performed on animal models and consequently have the disadvantage of not taking species differences into account. This type of assessment of recovery of sensation was used in the experiments described in this chapter.

5.1.3 Low Threshold Cutaneous Mechanoreceptors.

In the experiments described in this chapter, the skin was stroked to elicit a response in a variety of low threshold mechanoreceptors (Jackson & Diamond, 1981) and a response was recorded from those receptors lying in the peroneal cutaneous receptive field (see section 5.2.3). When recordings were made from whole nerve, as in the work presented here, the most prominent spikes that were recorded were from the stimulation of hair follicle receptors (Devor, Schonfeld, Seltzer and Wall, 1979) although Jackson and Diamond (1981) stress the importance of stimulation of the slowly-adapting type I mechanoreceptors or touch domes in their experiments involving mapping of cutaneous receptive fields. Lewin and McMahon (1991) showed that 82% of the myelinated afferents in the sural

nerve of rats had low threshold mechanosensitive fields and of these, 87% were rapidly adapting hair follicle afferents.

Hair follicle receptors are rapidly adapting mechanoreceptors which only respond to actual movement of the hair and not to static deformation of the skin. Hair follicle receptors are served by large myelinated fibres which may branch extensively to serve many hair follicles. The sensory endings vary in their arrangement around the hair follicle and can form a "basket-work" of nerve terminals orientated circumferentially and longitudinally above the root of the follicle (Iggo, 1977).

Touch domes are small elevations on the skin surface which contain a hair (tylotrich hair). In association with the hair are the mechanoreceptors themselves which are specialized epithelial cells called Merkel cells. Each touch dome is generally innervated by one myelinated axon which branches to serve the 20-50 Merkel cells (English, 1974). Movement of the hair causes mechanical deformation of the Merkel cells. It is thought that the Merkel cells release a neurotransmitter substance which causes depolarization of the axon. The ellicited reponse is an irregular, slowly adapting discharge. These slowly adapting cutaneous mechanoreceptors provide information about the mechanical conditions in the skin and the type I receptor responds when the skin is deformed (dynamic or first order derivative of skin displacement) and while the skin is held in its new position (static or zero order derivative of skin displacement) (Iggo, 1977).

5.1.4 Recovery of Sensory Function after Nerve Regeneration.

The success of regeneration of cutaneous sensory nerve fibres and their return to a similar distribution within their receptive field after an injury of the axonotmesis type has been reported to be good (Horch, 1979).

Horch and Lisney (1981) performed experiments where they crushed or transected the sural and posterior femoral cutaneous nerves of cats. Similarly to the findings presented in chapter 4, these authors found that by 6 months after nerve crush, the conduction velocity of the regenerated axons approached normal values, although after transection, the conduction velocities were only about 50% of control values. Horch (1979) mapped the distribution of slowly-adapting type I mechanoreceptors of the thigh, in cats, and investigated the pattern of reinnervation after nerve crush or transection of the femoral cutaneous nerve. After nerve crush the regenerating fibres returned to their old receptor sites and "essentially all type I fibres regenerated successfully". After nerve transection, the old pattern of reinnervation was not restored but old receptor sites were reinnervated. Horch (1979) suggested that this was evidence that sensory reinnervation after nerve crush is a very ordered process and is probably dependent on endoneurial tubes, distal to the site of the crush, acting as conduits.

After nerve section and repair, the anticipated success of recovery is much poorer owing to the 'cross-wiring' which inevitably occurs when regenerating axons traverse suture lines and travel down pathways that may lead them to inappropriate end-organs (see below). In order to appreciate the findings when cutaneous receptive fields are studied after different methods of nerve injury and repair, it is necessary to consider the factors affecting recovery of cutaneous innervation.

5.1.5 Factors Affecting Recovery of Sensory Function.

(a) Innervation density.

The extent of functional recovery of a cutaneous receptive field will depend not only on the number of fibres that regenerate successfully and return to reinnervate the skin but also on the number of receptors that each regenerated fibre reinnervates (Horch & Lisney, 1981). The aforementioned authors demonstrated that after transection of cutaneous nerves in cats, there was no loss of myelinated primary afferent fibres proximal to the injury and, in effect, all of the fibres were available for regeneration and reinnervation. They argued that the 25% of these fibres which did not regenerate across the unrepaired transection site could not account for the functional deficit and they suggested that this could be because individual axons served fewer receptors. This reduction of innervation density could result in impaired recovery of function. In contrast, Jabeley, Burns, Orcutt and Bryant (1976) showed a poor correlation between receptor density and recovery of function. Horch (1979) investigated the reinnervation of type I cutaneous mechanoreceptors after nerve crush and nerve transection (without repair). In his controls, he found that the average number of type I cutaneous mechanoreceptors (touch domes) innervated by each type I sensory neuron in cat cutaneous nerves was 2.4-2.6. After nerve crush, he found that "essentially all type I fibres successfully regenerate" and each regenerated type I sensory neuron innervated, on average, 2.2 type I cutaneous mechanoreceptors. After nerve transection, only 60% of type I fibres regenerated back to the skin and each fibre reinnervated half the number of domes that a fibre in a control animal innervated (i.e. 1.3 receptors).

Sanders and Zimmermann (1986) demonstrated the rapid return of function of low-threshold cutaneous mechanoreceptors to rat glabrous skin after nerve crush. The responsiveness of rapidly and slowly adapting mechanoreceptors recovered gradually with time. They suggested that the gradual increase in responsiveness after regeneration was due to more end-organ/axon connections having been made. Greater responsiveness to stimuli may result from the summation of impulses originating from different receptors served by the same fibre.

(b) Aberrant innervation of receptors.

As a result of fibres regenerating across suture lines, inappropriate connections between regenerated nerve axons and receptors may occur. Not only may the newly formed axon/receptor connections function abnormally but the normal somatotropic organization of fibres is also likely to be disrupted. The inability of the CNS to 'cope' with the new pattern of innervation may be such that the perception of recovery is poorer than would be expected from the degree of reinnervation.

The idea that regenerating nerve fibres selectively 'choose' the pathway down which they regenerate is a controversial one. The reinnervation of end-organs by regenerating axons may be a random process, alternatively, specificity may occur at one of three levels described by Rath and Green (1991):

(1) Tissue specificity.

This type of specificity describes the growth of axons towards nerve rather than other tissue e.g. a transected nerve will tend to grow towards its distal stump rather than into the surrounding non-neural tissue even when the nerve has not been repaired (Horch, 1979).

(2) End-organ specificity.

This type of specificity describes the preference of sensory/motor axons to grow towards the end-organ that they initially innervated (Brushart & Seiler, 1987).

(3) Fascicular specificity.

This occurs when axons from one fascicle discriminate between fascicles serving particular anatomical regions and preferentially regenerate down their original fascicle (Politis, 1985). Rath and Green (1991) investigated the regeneration of rat sensory axons from the transected lateral cutaneous nerve of the thigh, through a Y-shaped freeze-thawed skeletal muscle autograft, towards the original distal stump and to the distal stump of the transected saphenous nerve. They demonstrated that the axons showed no preference to return to their original receptive fields and, therefore, regenerating sensory axons do not show topographical specificity.

In addition, to these three types of specificity, a fourth could be added:

(4) Receptor specificity.

This type of specificity describes the selective growth of axons towards the receptors that they originally innervated. Burgess and Horch (1973) investigated the neuronal and receptor properties of sensory neurons after transection and regeneration of the sural nerve in cats. They found that the conduction of each peripheral neuron was appropriate for the mechanosensory function of its receptor and this led them to suppose that some sort of target specificity of regeneration had occurred. Alternatively, Weiss (1936) proposed that the regeneration of fibres after nerve transection was random and that it was the connections in the CNS that were rearranged. Horch (1976) demonstrated that, after transection and regeneration of the sural nerve in cats, no apparent changes in the

arrangement of ascending collaterals in the dorsal columns occured, thus substantiating the proposals of Burgess and Horch (1973) that selectivity of regenerating sensory axons towards specific receptors had occurred. However, aberrant reinnervation of receptors also takes place. Lewin and McMahon (1991) "cross-anastomosed" the proximal stump of the transected gastrocnemius muscle nerve with the distal stump of the transected sural nerve in the rat. Their results showed that muscle afferents could reinnervate skin mechanoreceptors but the newly formed afferents retained some of their old characteristics *i.e.* they showed a predominance of slowly-adapting responses. The fact that the newly formed afferents retained some of their old characteristics, in contrast to the findings of Burgess and Horch (1973), who found that the newly formed afferents in their experiments had appropriate properties for the receptor type, suggested that receptor specificity of regeneration does take place when it is possible for it to do so. In contrast, McMahon, Lewin, Anand, Ghatei and Bloom (1989) showed that regenerated afferents were able to take on the characteristics of the new end-organ. They investigated the regenerated fibres of the gastrocnemius muscle nerve, after it was "cross-anastomosed" with the distal stump of the transected sural nerve. They found that the levels of substance-P and calcitonin gene-regulated peptide, which are higher in cutaneous nerves than in muscle nerves, were increased in muscle afferents when they were reconnected to skin.

Horch (1979) demonstrated that after nerve transection, regenerating type I nerve fibres preferentially returned to old slowly-adapting type I mechanoreceptor sites. In later work, Horch (1982) cauterized sites of slowly-adapting type I mechanoreceptors and found that type I nerve fibres regenerated preferentially towards uncauterized receptor sites. This led him to the conclusion that the reinnervation of old receptor sites by type I nerve

fibres was due to the intrinsic properties of these sites rather than the guidance of the said fibres down old type I Schwann tubes. Should these findings be accurate, the distance of the site of the injury from the end-organ is undoubtably crucial to the recovery.

(c) Changes in normal functioning of receptors.

Functioning of reinnervated receptors or the functioning of newly formed receptors may be different from controls. Munger (1988) stated that the process of nerve regeneration can never be the same as the process of nerve development and, therefore, the redifferentiation of sensory corpuscles after nerve injury and regeneration should never be expected to be normal. In his review article he reported ultrastructural abnormalities in Merkel terminals and hair receptors (among others) after reinnervation. Abnormal structure does not necessarily mean abnormal function, however, reports have been made of raised mechanical thresholds of cutaneous mechanoreceptors (Munger, 1988; Lewin & McMahon, 1991; Sanders & Zimmerman, 1986).

(d) Poor recovery of sensory receptive field.

The experiments of this chapter involved mapping the area of the peroneal cutaneous receptive field in rats. Gutmann, Guttmann, Medawar and Young (1942) observed that the recovery of the peroneal nerve receptive field in the rabbit progressed in a proximo-distal direction. The skin on the dorsum of the foot recovered last. Similarly, Sanders & Zimmermann (1986) found that the reinnervation of mechanoreceptors of rat glabrous skin by regenerating axons shows a proximal-to-distal shift as the receptive field enlarges with time.

Findlater, Reichert and Glasby (1990) investigated the recovery of peroneal cutaneous receptive fields, in the rat, 250 days after muscle grafting the sciatic nerve. They did not find any significant difference in receptive field area between control and regenerated nerves. Lewin and McMahon (1991) recorded the receptive field areas of the afferents in the regenerated rat gastrocnemius muscle nerve which had been sutured to the distal stump of the transected sural nerve. Although the nerve to the gastrocnemius had fewer fibres than the sural nerve, these authors found that the receptive field areas did not differ significantly in size from those of control sural nerve or transected and sutured sural nerve.

Axons regenerating across nerves that have been transected have been reported to innervate receptors which are spatially separated (Horch & Lisney, 1981). These fibres with split cutaneous fields are unlikely to have affected the results of these experiments (see section 5.4). Changes in cutaneous receptive fields have been recorded as a result of sprouting from neighbouring cutaneous nerves: Sprouting of intact and regenerating nerve fibres.

Ramon y Cajal (1928) was the first to suggest that growth promoting factors produced in the target tissue influenced the sprouting of nerve fibres in the periphery during development and to prevent excessive sprouting, a 'neutralizing' factor was produced by the nerve sprouts themselves. By this mechanism, the end-organ could be adequately, but not excessively, innervated.

Some research has indicated that sprouting also occurs after development. Sprouting from intact nerve in response to denervation of an adjacent mechanosensory field has been termed 'denervation sprouting'. Devor *et al.* (1979) showed an expansion of the functional distribution of high threshold mechanoreceptors from the saphenous nerve in rats after nerve

ligation or crush of the sciatic nerve. These authors believed the expansion to be due to invasion of sciatic 'territory' by collateral out-growths of neighbouring saphenous axons. They also noticed a retraction of the expanded functional distribution of the saphenous nerve as the sciatic nerve regenerated back to its original boundaries. Collateral reinnervation has also been observed in the salamander hind limb and from their experiments, Diamond, Cooper, Turner and Macintyre (1976) proposed that each nerve has a territory of its own which they called 'body space'. These authors showed that after denervation of an area of skin, sprouts from the intact nerve innervating the adjacent area "break out of their body space" but this seemingly depended on which nerve was being investigated and tended to occur more readily in the distal part of the limb.

Both Devor *et al.* (1979) and Diamond and Jackson (1978) failed to encounter collateral spread of low threshold receptive fields. The latter authors investigated the expansion of low threshold mechanosensory fields of intact nerve after the denervation of adjacent skin, in young and adult rats and rabbits. Only in young rats (earlier than 21 days after birth) was collateral sprouting of intact nerve observed in response to the denervation of adjacent skin, resulting in the formation of abnormally large mechanosensory fields of the intact nerve. Jackson and Diamond (1981) noticed that low threshold mechanosensory nerves from intact nerves could only invade adjacent areas of denervated skin in rats younger than a critical age (20 days). Kinnman and Aldskogius (1986) also reported similar findings of collateral sprouting of intact saphenous nerve into the denervated glabrous skin in neonatal rats. Although they also reported limited collateral sprouting in adult rats, only thin saphenous nerve axons ventured outside the normal saphenous territory. In another paper, Wiesenfeld-Hallin, Kinnman and Aldskogius (1988) described findings of a

considerable overlap between the cutaneous receptive fields of the C-fibres of the various nerves serving the hind limb of the rat and suggested that the 'denervation sprouting' observed by some authors could be explained by the uncovering of this normal overlap of receptive fields.

Aguilar, Bisby, Cooper and Diamond (1973) proposed a mechanism to explain nerve sprouting, very similar to that of Ramon y Cajal (1928; see above), such that target tissues are continually producing factors which stimulate nerve sprouting but these factors are somehow neutralized by other factors released from the fibres themselves and that these "neutralizing" factors are delivered by axonal transport. Alternatively, the factors produced in the tissue which stimulate nerve sprouting may be removed by retrograde axonal transport so that, in normally innervated tissue, the production and removal of factors are balanced and no expansion or retraction of the cutaneous field occurs.

Doubleday and Robinson (1992) ligated the inferior alveolar nerve in rats and looked at the collateral innervation by C-fibres from the contralateral nerve. They discovered that immunization against NGF prevented collateral reinnervation, leading them to the conclusion that NGF is essential for the development of collateral reinnervation and may be the initiating stimulus or factor. Accumulation of NGF in denervated tissue may occur if there is an increased production of NGF and/or the removal of NGF by retrograde axonal transport is reduced. Diamond, Coughlin, Macintyre, Holmes and Visheau (1987) also found that collateral innervation from nociceptive axons in rats was prevented by administering NGF antiserum, although regeneration of axons was unaffected.

Diamond *et al.* (1976) demonstrated that regenerating nerve fibres were not governed by the same limitations of 'body space' (described above) and regenerating fibres which were once contained within one 'body

space' grew to reinnervate 'alien' territory. Jackson and Diamond (1981) confirmed the theory that regenerating nerve fibres do not respect the 'body space' rules, in their work on rats. When severed nerve fibres regenerated, they functionally displaced those fibres that had previously entered the area as collateral sprouts from intact nerve. Kinnman and Aldskogius (1986) found that "regenerating sensory axons in adult rats seem to have a greater capacity for collateral sprouting than intact axons".

Although some degree of specificity of growth probably occurs during regeneration, the success of recovery of function probably depends a great deal on both surgical technique and luck. Fascicular repair may be advantageous in multifascicular nerves, if bundles can be matched correctly; aside from this the surgeon must simply perform the operation and hope that recovery is good.

5.2 Materials and Methods.

5.2.1 Exposure of the Common Peroneal Nerve.

150 days after operation, each rat was anaesthetized (chapter 2.3) and kept warm on an electrically heated blanket. The left hindquarter was shaved using electrically operated clippers.

The common peroneal nerve on both sides was identified by first locating the sciatic nerve in the way described in chapter 2.4, and following it distally until its branching point where the common peroneal and tibial nerves originate. This approach reduced the probability of disturbance of the receptive field at the point of incision. In the 1 cm muscle graft groups the sciatic nerve proved more difficult to find on the grafted side owing to the similarity in appearance of graft, connective tissue and muscle. However, if the incision was extended it was possible to find the peroneal and tibial nerves as they passed distally from the graft.

The peroneal nerve was carefully freed from the surrounding connective tissue, with the aid of an operating microscope (Weck Fibermatc 0902A1, Long Island City, New York). Fine forceps were used to remove as much of the epineurium from around the neve as possible. This increased the signal-to-noise ratio in subsequent electrophysiological recordings.

5.2.2 Electrophysiological Apparatus.

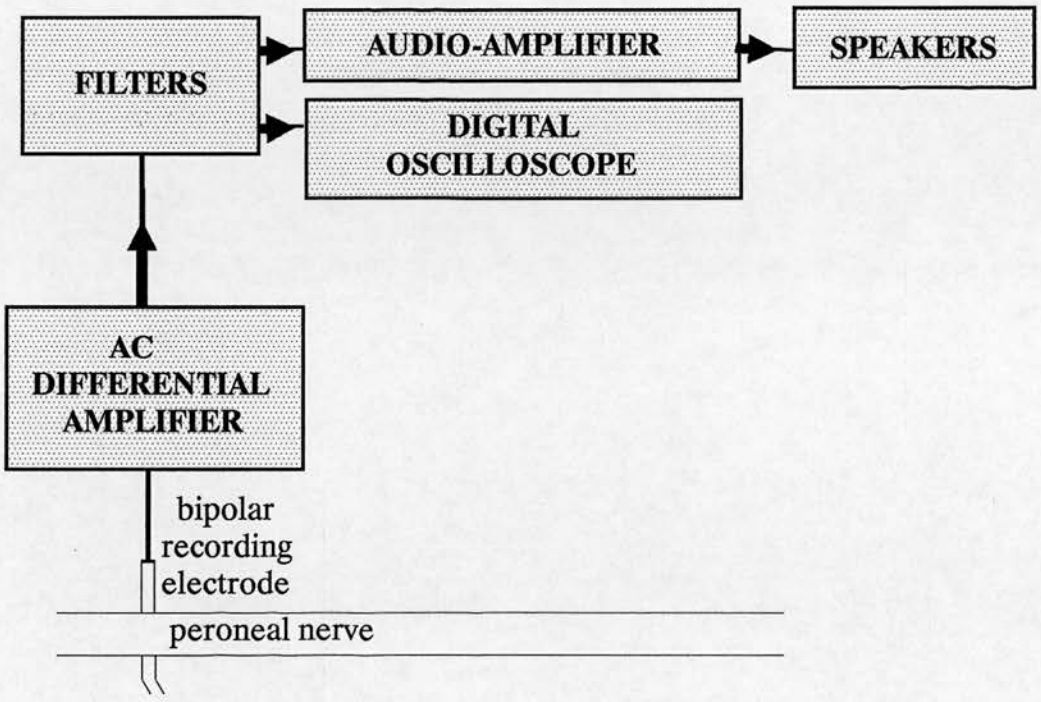
The arrangement of the electrophysiogical apparatus that was used in the experiments described here is shown in figure 5.1. The peroneal nerve was divided, as near as possible to the point of branching from the sciatic

nerve. This allowed the longest length of the peroneal nerve, as possible, to be freed for placing over to the poles of the recording electrode. In this way, stretching of the peroneal nerve was prevented. The tibial nerve was also divided. The distal stump of the peroneal nerve was placed over a silver wire, bipolar, low impedance recording electrode and a silver wire earth electrode was passed through the muscle underlying the nerve. Both electrodes were connected to the appropriate terminals of a Neurolog NL104A AC amplifier (Digitimer Ltd, Welwyn Garden City, U.K.). The recorded signal was then passed through a Neurolog NL125 filter (Digitimer Ltd). The output from the filter was amplified by a Neurolog NL120 audio-amplifier (Digitimer Ltd) and was simultaneously displayed on a channel of a Gould 4074 digital storage oscilloscope (Gould Electronics Ltd, Ilford, U.K.). It was thus possible to hear the amplified electrical response to mechanical stimulation and to see volleys of spikes displayed on the oscilloscope, both produced by mechanical excitation of low-threshold mechanoreceptors in the cutaneous receptive field of the common peroneal nerve.

5.2.3 Measurement of Receptive Field Area.

The cutaneous receptive field of the common peroneal nerve was identified on the operated and control sides by listening to the afferent impulses evoked by brushing or tapping the leg with a fine probe. In this way the edges of the receptive field, which was the outer limit where responses were obtained, could be mapped out with a permanent ink pen. When commencing the experiment the filters were kept as wide as possible; then by simultaneously stroking the leg and adjusting the filters, it was possible to improve the signal-to-noise ratio. The afferent impulses evoked by brushing the leg were then easily identified.

Figure 5.1 Diagram of the electrophysiological apparatus used to rcord the peroneal cutaneous receptive field



5.2.4 Removal of Skins.

One problem encountered was the tendency for skin to shrink when removed from the animal as a result of its elasticity. This affected the subsequent laying out of skins on cork boards, to the correct area. The problem was overcome as follows:

before removing the skins, the receptive field areas, previously outlined in permanent ink, were marked with 3/0 Vicryl sutures (Ethicon Ltd, Suture Division, Edinburgh, U.K.). This was achieved by knotting the suture thread at the ink line and then knotting it again at a line diagonally across from the first knot, keeping the suture thread taught. This was repeated two

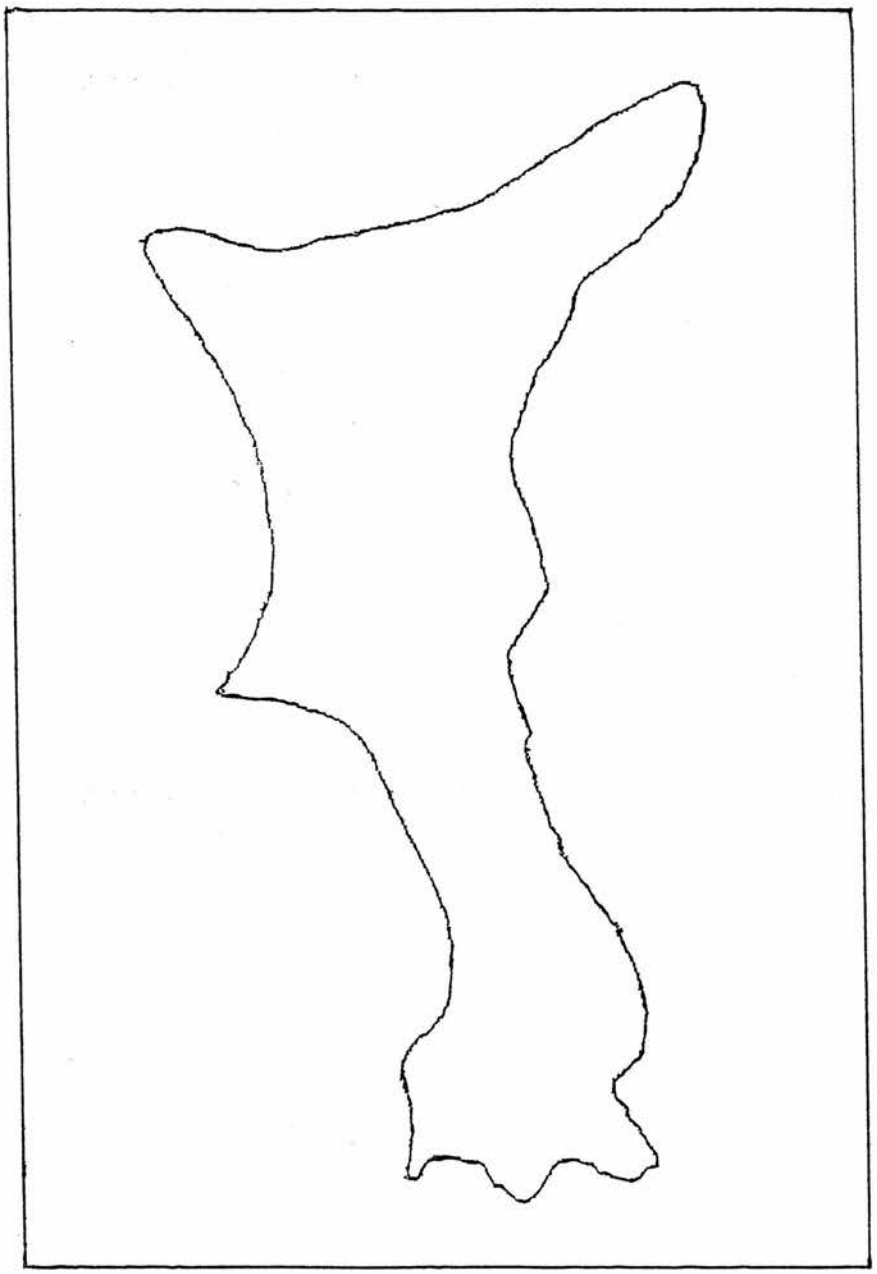
or three times. A scalpel was used to cut around each skin at the permanent ink lines. The skin was then removed and laid out on a cork board so that the suture thread was taught. This method ensured that the skins were stretched to the correct area.

5.2.5 Morphometry of Cutaneous Receptive Field Area.

The cork boards and skins were transferred to a photomacrography stand. The skins were placed under a television camera fitted with a Cannon 100 mm, F 3.5 macro lens and were illuminated from above. The output from the camera was connected to the Vids III image analysis system (Analytical Measuring Systems Ltd, Saffron Walden, U.K.). The system is described in chapter 4.2.9. The skins were imaged by the television camera and were displayed on the monitor. The VIDS III was calibrated to correspond with the magnification of the tissue by the macro lens in cm per pixel.

By moving the cursor across the digitizing tablet, a current is induced in a coil within the cursor and the system produces a corresponding movement of a cursor on the monitor screen. In this way, it was possible to draw around the image of the cutaneous receptive field which was displayed on the monitor screen. The VIDS III system calculated the encircled areas. A typical receptive field is shown in figure 5.2.

Figure 5.2 A trace obtained using the VIDS III image analysis system to encircle the cutaneous receptive field area of a rat peroneal nerve. Magnification X1



5.3 Results.

5.3.1 Results from Peroneal Nerve Cutaneous Field Experiments.

Of the 10 groups of rats used in the experiments described in this chapter, 9 of them contained 5 rats. The exception was the non-diabetic nerve graft group which contained 7 animals. A table of the values of left, right and the ratio of left/right peroneal cutaneous receptive field areas for each animal is shown in appendix 13.

As discussed in chapter 3, the diabetic rats had a lower mean weight and were, on average, smaller in size than the non-diabetic animals. Inevitably, the size of the peroneal cutaneous receptive field of the diabetic rats was smaller than the same in the non-diabetic rats. The mean receptive field area of the right unoperated sides in the non-diabetic animals was 10.05 cm^2 (SD = 1.45, n = 27). The mean receptive field area of the right unoperated sides in the diabetic animals was 8.89 cm^2 (SD = 1.00, n = 25). It was, therefore, considered necessary to compare the recovery of receptive field area on the operated side as a fraction of the receptive field area on the unoperated side (receptive field ratio). This manoevre compensates for differences in the size of the receptive fields between diabetic and non-diabetic rats which were due to the direct effect of the diabetic state. This compensation is demonstrated by the fact that the mean peroneal cutaneous receptive field ratio for both groups of controls approximated to unity (see tables 5.1 and 5.2). The mean receptive field ratio was 1.02 (SD = 0.18, n = 5) for the non-diabetic controls and 1.00 (SD = 0.09, n = 5) for the diabetic controls.

A plot was made of the residuals of receptive field area against their fitted values (see chapter 4.3.1) and from this plot, it was found that the variance of each experimental group was similar. Normal and half-normal plots were made from the data of receptive field ratio (see chapter 4.3.1). One outlier was found which belonged to the diabetic nerve graft group. This data point was ignored in the subsequent statistical tests as it did not fit the normal distribution of the other data points. The process of making normal and half-normal plots was repeated. It was found that the remaining data were normally distributed and thus provided justification for the use of parametric tests in the analysis of the recorded values of receptive field ratio (RF ratio).

The values of the mean, standard deviation, standard error of the mean and the coefficient of variation of receptive field ratio for the non-diabetic rats is shown in table 5.1, and the equivalent for the diabetic rats are shown in table 5.2. Figure 5.3 is a bar chart showing the mean receptive field ratio for each of the non-diabetic experimental groups and figure 5.4 shows the equivalent for the diabetic experimental groups. From these tables and figures, it can be seen that the degree of recovery of receptive field area varies inversely with the severity of the injury in most cases and that, in each type of injury, the recovery was poorer in the diabetic groups.

Figure 5.3 Bar chart showing the mean receptive field ratio (± SEM) for each of the non-diabetic groups of animals (n = 5 for each group)

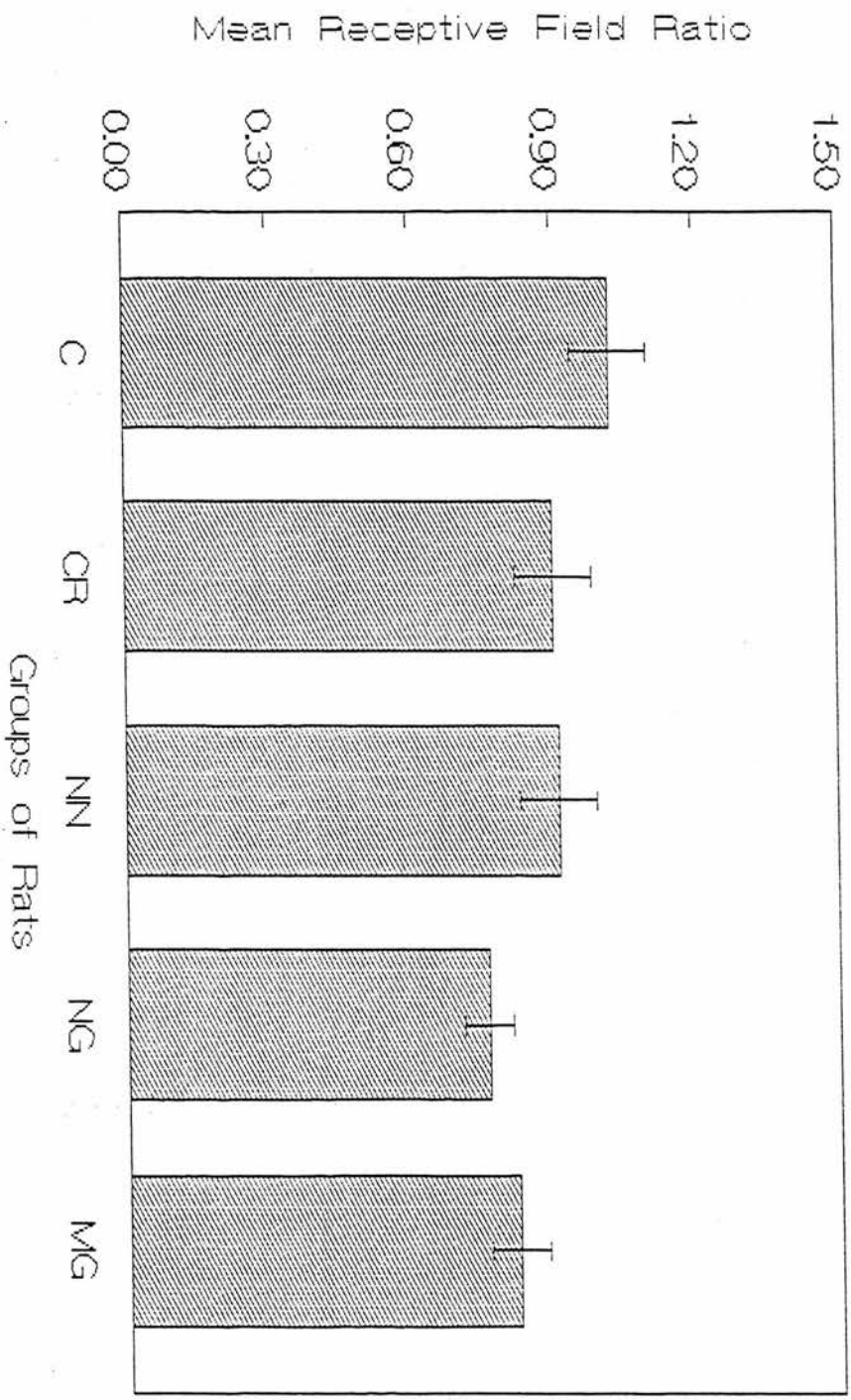


Figure 5.4 Bar chart showing the mean receptive field ratio (± SEM) for each of the diabetic groups of animals (n = 5 for each group)

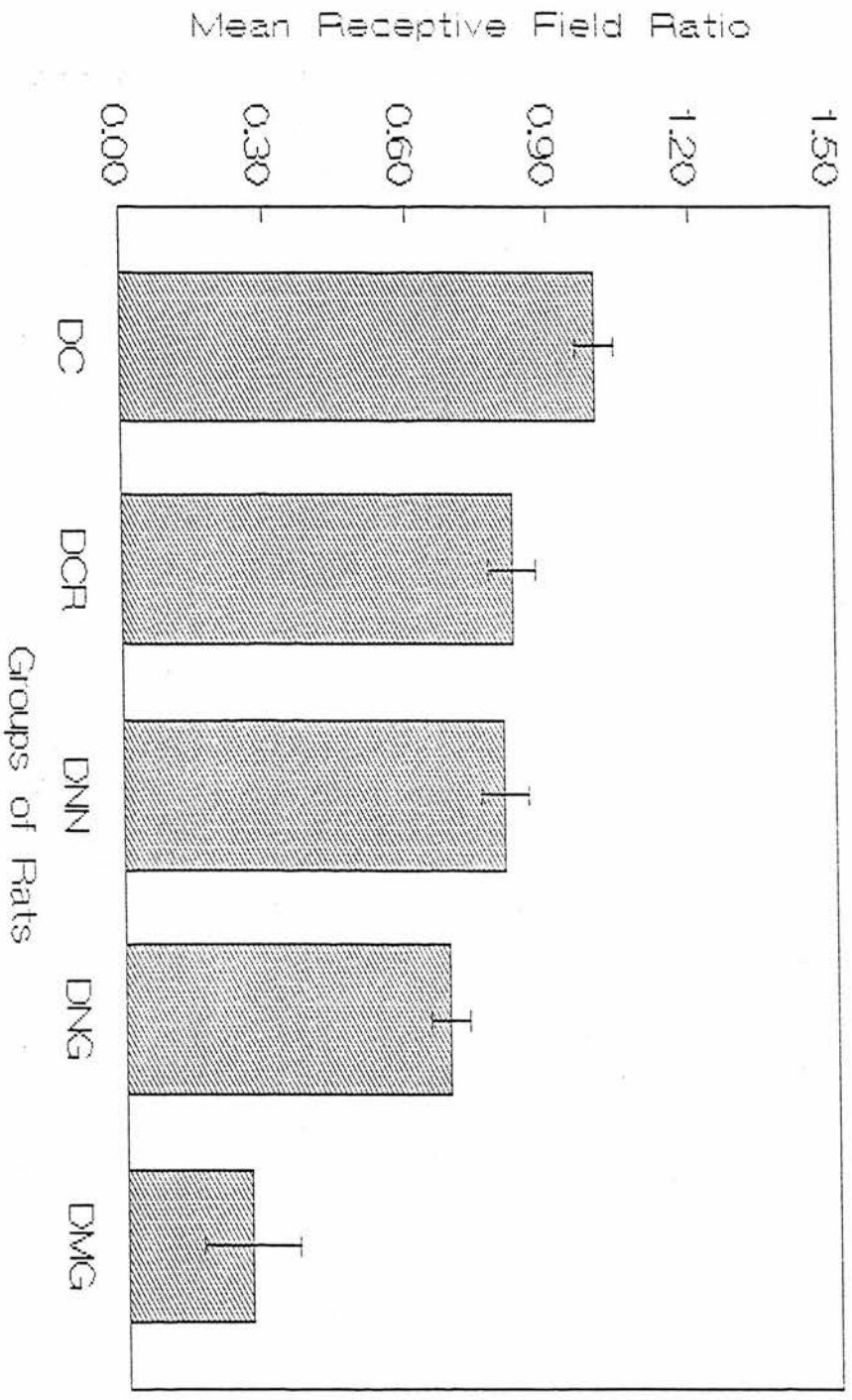


Table 5.1 Values of the mean, standard deviation, standard error of the mean and coefficient of variation of the ratio of left/right peroneal cutaneous receptive field for non-diabetic animals

RAT GROUP	n	m	SD	SEM	c.v.
C	5	1.02	0.18	0.08	17.71
CR	5	0.90	0.18	0.08	19.45
NN	5	0.91	0.18	0.08	19.24
NG	7	0.76	0.10	0.05	13.70
MG	5	0.82	0.14	0.06	17.52

Table 5.2 Values of the mean, standard deviation, standard error of the mean and coefficient of variation of the ratio of left/right peroneal cutaneous receptive field for diabetic animals

RAT GROUP	n	m	SD	SEM	c.v.
DC	5	1.00	0.09	0.04	9.38
DCR	5	0.82	0.12	0.05	14.37
DNN	5	0.80	0.11	0.05	13.67
DNG	4	0.68	0.10	0.04	14.85
DMG	5	0.26	0.22	0.10	84.42

Results from significance tests on the values of receptive field ratio.

a) F test for variance.

The value of F obtained from performing the F test on all values of receptive field ratio in all groups was $F = 8.69$, d.f. = 11, 59. This was significant ($p < 0.01$) and the null hypothesis, asserting that there is no difference in receptive field ratio between the different experimental groups, was rejected.

b) Student's t tests on the means of receptive field ratio.

Non-diabetic rats.

Table 5.3 shows the values of t and the significance of t tests, at the 5% and 1% levels, performed on the means of receptive field ratio in non-diabetic animals. The mean receptive field ratio was less than 1 in all of the operated non-diabetic groups which suggests that an incomplete recovery is obtained by 150 days. The value of t obtained when comparing the control group with nerve graft and muscle graft groups approached significance, and may have proved to be significantly different from each other had the size of the groups been larger. However, with the size of the groups used, none of the operated groups was significantly different from the control group, nor were they significantly different from each other, at the 5% level.

Table 5.3 Table of t values and significance levels for the values of left/right peroneal cutaneous receptive field in non-diabetic animals (Student's t test)

	C	CR	NN	NG	MG
C	_	t=1.25 NS	t=1.09 NS	t=2.88 NS	t=2.07 NS
CR	_	_	t=-0.15 NS	t=1.54 NS	t=0.83 NS
NN	_	_	_	t=1.70 NS	t=0.98 NS
NG	_	_	_	_	t=-0.64 NS
MG	_	_	_	_	_

Diabetic rats.

Table 5.4 shows the values of t and the significance of t tests, at the 5% and 1% levels, performed on the means of receptive field ratio in diabetic animals. A very different pattern of significance was obtained from that seen in non-diabetic animal groups (see table 5.3). Although the mean of the receptive field ratio was less than 1 in all of the operated groups (table 5.2), the mean receptive field ratio of the crush, nerve-to-nerve suture and nerve graft groups was not significantly smaller than that of the control group ($p > 0.05$). The mean receptive field ratio of the muscle graft group was significantly smaller than that of the control group at the 1% level.

The mean receptive field ratio of the diabetic muscle graft group was 0.26 (SD = 0.22, n = 5). This was significantly smaller than the mean receptive field ratio of the crush and nerve-to-nerve suture groups at the 1% level. The mean receptive field ratio was also significantly smaller than the mean receptive field ratio of the nerve graft group at the 1% level, even though the number in the diabetic nerve graft group was only 4. Using the method described in chapter 5.2 for measuring the peroneal cutaneous receptive field, there was no detectable receptive field in 2 out of the 5 animals in the muscle graft group.

After nerve injury and repair, the peroneal cutaneous receptive field had not returned to control values by 150 days. The return of receptive field area was inversely proportional to the severity of the injury, however, the recovery after muscle grafting was particularly poor.

Table 5.4 Table of t values and significance levels for the values of left/right peroneal cutaneous receptive field in diabetic animals (Student's t test)

	DC	DCR	DNN	DNG	DMG
DC	_	t=1.84 NS	t=2.04 NS	t=3.10 NS	t=7.71 p<0.01
DCR	_	_	t=0.20 NS	t=1.37 NS	t=5.68 p<0.01
DNN	_	_	_	t=1.17 NS	t=5.48 p<0.01
DNG	_	_	_	_	t=3.99 p<0.01
DMG		_	_	_	_

Diabetic *v* non-diabetic rats.

Table 5.5 shows the t values and levels of significance for the t tests performed on similar experimental groups in the diabetic and non-diabetic states. Each experimental diabetic group regained a mean receptive field ratio smaller than the equivalent non-diabetic group mean. The mean receptive field ratio was significantly smaller only in the diabetic muscle graft group as compared to the non-diabetic muscle graft group ($p < 0.01$). It seems that the diabetic state did make a difference to the recovery of cutaneous receptive field area but the difference was only significant in the group where the most severe form of injury was repaired with a muscle graft.

Table 5.5 Table of t values and significance levels for the values of left/right peroneal cutaneous receptive field in non-diabetic animals v diabetic animals (Student's t test)

	C	CR	NN	NG	MG
DC	t=0.19 NS	–	–	–	–
DCR	–	t=0.78 NS	–	–	–
DNN	–	–	t=1.14 NS	–	–
DNG	–	–	–	t=0.81 NS	–
DMG	–	–	–	–	t=5.63 p<0.01

5.4 Discussion.

In the experiments presented here, a response was ellicited from a variety of low-threshold mechanoreceptors by stroking the skin (Jackson and Diamond, 1981; see chapter 5.1.3). The most prominent of the electrical responses that were recorded was from hair follicle receptors and the slowly-adapting type I cutaneous mechanoreceptors (Devor *et al.*, 1979; Lewin & McMahon, 1991). Therefore, it should be noted that the recovery of function that was investigated in these experiments related to the return of the response of mechanoreceptors to low-threshold stimuli. It has been reported that the recovery of painful sensation (high-threshold receptors) progresses at a faster rate than the response to light touch (low-threshold receptors)(Gutmann *et al.*, 1942).

5.4.1Receptive Field Ratio.

The receptive field ratio of the non-diabetic and diabetic control groups approximated to unity in both cases (tables 5.1 and 5.2). This result suggests that the peroneal cutaneous receptive fields of the legs of rats are related in size. Wiesenfeld-Hallin *et al.* (1988) also demonstrated a highly symmetrical pattern of distribution of the cutaneous receptive fields of the hindlimbs of rats. The receptive fields of unoperated nerves on contralateral sides have been used as control values by many researchers (Devor *et al.*, 1979: Wiesenfeld-Hallin *et al.*, 1988; Diamond *et al.*, 1976, 1987; Findlater *et al.*, 1990). It is in the light of these facts that the receptive field ratio was considered a valid measurement to make. Diamond *et al.* (1976) showed that although the mechanosensory fields of the three segmental nerves

which supply the salamander hind limb are symmetrical between limbs, the areas vary from animal to animal. Differences in the size of mechanosensory fields from one animal to another are only to be expected considering that animals themselves vary in size. Despite the similarity in age, the variation in weight and size of the animals used in the work presented here was particularly large, owing to the fact that the diabetic animals weighed considerably less than the non-diabetics (chapter 3.3.1). The use of receptive field ratio in statistical tests was, therefore, deemed not only valid, but necessary.

5.4.2 Recovery of Receptive Field Ratio.

The mean receptive field ratio was smaller in all of the operated groups of non-diabetic animals when compared to the control group; however, statistical tests showed no significant difference between any combination of pairs of groups ($p > 0.05$; tables 5.1 and 5.3). Similar findings were recorded in the diabetic animals although the values of mean receptive field area were smaller in the diabetic groups when compared to the equivalent non-diabetic groups (table 5.2). The mean receptive field ratio attained after muscle grafting was significantly smaller than the mean receptive field ratio of any other diabetic group ($p < 0.01$; table 5.4).

(a) Recovery after nerve crush and nerve-to-nerve suture.

150 days after nerve crush and nerve-to-nerve suture, the mean receptive field ratio in the non-diabetic rats was 0.9 and 0.91, respectively. The mean receptive field ratio of the nerve crush and nerve-to-nerve suture groups in the diabetic rats was also very similar: 0.82 and 0.8, respectively. Although the values were smaller in the diabetic animals, the similarity in the size of mean receptive field ratio, after the two types of injury and repair,

show that there was no real difference in recovery 150 days after nerve crush or nerve-to-nerve suture. Horch (1979) found that 6 months after crushing the femoral cutaneous nerve in cats, "essentially all type I fibres regenerated" and they returned to their old receptor sites. Other authors have also demonstrated a good return of function after nerve crush (Gutmann *et al.*, 1942; Horch & Lisney, 1981), although the recovery after nerve transection was not so impressive (Horch & Lisney, 1981). The similarity in recovery of receptive field area between the nerve crush and nerve-to-nerve suture groups, reported here, might be because the recovery after nerve crush was unusually poor or that the recovery after nerve-to-nerve suture was particularly good. Recovery could be unexpectedly poor after nerve crush if the injury inflicted was more severe than the intended axonotmesis (see chapter 4.4.3). The recovery after nerve transection was better than would be expected from the reports of Horch and Lisney (1981), however, in the experiments of these authors, the transected nerve was not repaired. Gutmann *et al.* (1942) showed good recovery after nerve-to-nerve suture (see below).

It is possible that further increases in the size of the peroneal cutaneous receptive field, in the experiments reported here, would have happened had the recovery period been longer. Gutmann *et al.* (1942) investigated the rate of return of painful sensation to the rabbit hindlimb after crushing the peroneal nerve. They found that the time required for complete sensory recovery, when the peroneal nerve regenerated over a distance of approximately 25 cm, was around 100 days, and they calculated an average rate of recovery of 3.39 mm day^{-1}. They found that the rate of shrinkage of analgesic areas of the foot was 2.1 mm day^{-1} after nerve crush and 1.6 mm day^{-1} after nerve-to-nerve suture. Although the species used in the experiments of this thesis was the rat, it is not unreasonable to presume that

complete sensory recovery had been attained considering that the nerves had shorter distances over which to regenerate than the animals of the aforementioned authors and the animals were allowed 150 days to recover.

A possible reason for the incomplete recovery seen in the nerve crush (or indeed any other experimental group) is the invasion of the peroneal receptive field by collateral sprouts from neighbouring intact nerves. This invasion, however, is highly unlikely considering that the only report of expansion of receptive fields, in adult rats, were high-threshold mechanoreceptors (Devor *et al.*, 1979; see chapter 5.1.5). Expansion of receptive fields of low-threshold mechanoreceptors have been reported in rats that were younger than 20 days at the time of denervation (Diamond & Jackson, 1978; Jackson & Diamond, 1981) but the rats used in the experiments reported here were all at least 10 weeks old at the time of operating on the sciatic nerve. Invasion of the peroneal receptive field by neighbouring nerves, in the animals used in the work of this thesis, is only likely to have occurred if these neighbouring nerves were themselves severed at the time of operation. It was demonstrated by Diamond *et al.* (1976) that regenerating nerve fibres were not limited by the constraints of 'body space' (see chapter 5.1.5) and that regenerating nerve fibres could reinnervate the 'territory' of another nerve if the said 'territory' had been denervated. Similar findings were reported by and Jackson and Diamond (1981) and Kinnman and Aldskogius (1986). It is quite probable that such an invasion of the peroneal receptive field occurred in the experiments presented here because the sural nerve was frequently denervated at the time of operating on the sciatic nerve. The sural nerve leaves the sciatic nerve proximal to the peroneal-tibial bifurcation but in the operated nerves, the site of crush, suture or graft was proximal to the point at which the sural

branches from the sciatic. The regeneration of sural fibres into peroneal nerve territory would explain the poorer recovery of receptive field area after nerve crush than might be expected (see above).

(b) Recovery after grafting.

The recovery of receptive field area in the non-diabetic animals after nerve graft and muscle graft was worse in both cases than after nerve crush or nerve-to-nerve suture, although not significantly so ($p > 0.05$). Findlater *et al.* (1990) showed the percentage recovery of the rat peroneal receptive field area, 250 days after muscle grafting the sciatic nerve, to be 83.16%. This compares well with the results of the work presented here which showed recovery to be 82%. The fact that Findlater *et al.*, (1990) showed a very similar level of recovery after 250 days suggests that it is likely that the recovery of the response to low-threshold stimuli had reached a maximum by 150 days.

The same arguments for a less than complete functional recovery apply to these repair types (see above), as they do to nerve crush and nerve-to-nerve suture. The slightly poorer recovery seen after grafting than after nerve crush or nerve-to-nerve suture could be due to an additional time delay in overcoming a second suture line. Additionally, there may be shrinkage or degeneration of endoneurial tubes (Thomas, 1964) in the periphery which would limit the progession of pioneering axons towards their end-organs and would hinder receptor-axon reconnection. Degeneration of endoneurial tubes could account for the non-linear rate of regeneration of peripheral nerve that is encountered in man; regeneration rates in distal parts are decreased (Lundborg, 1988). A third possibility for the poorer recovery seen after grafting as compared to nerve crush and nerve-to-nerve suture, is that specificity of regeneration is reduced over two

suture lines. Horch (1982) proposed that the reinnervation of old receptor types by type I nerve fibers was due to axons responding to tropic/trophic influences from these old sites. If factors released from end-organs encourage the directional growth of regenerating axons, additional suture lines may interfere with this directional regeneration.

(c) Recovery of receptor density.

The mapping of receptive fields was found to be somewhat more difficult, though not unduly so, from the nerves of those animals that had suffered injury of the neurotmesis type, as compared with control nerve and nerve that had been crushed. In the experiments presented here, the skin was stroked to stimulate low-threshold mechanoreceptors and the electrical signals ellicited in the peroneal nerve were amplified by an audio-amplifier (see chapter 5.2.3). The recordings were fainter when they were made from nerve that had regenerated after nerve transection. This may be because the reinnervation of receptors was poorer after injuries of the neurotmesis type and the subsequent decrease in receptor density made recordings more difficult. Horch and Lisney (1981) proposed that the reason for the functional deficit they observed in the cutaneous nerves of their cats after nerve transection, was that regenerated axons served fewer receptors. In contrast, Jabeley *et al.* (1976) showed a poor correlation between receptor density and functional recovery. However, Horch (1979) showed that nerve fibres that had regenerated after nerve section typically innervated half the number of type I cutaneous mechanoreceptors of control fibres and fibres that had regenerated after crush.

Horch (1979) demonstrated that the old pattern of reinnervation of type I cutaneous mechanoreceptors is restored after nerve crush injury of the femoral cutaneous nerve in cats. He suggested that this was evidence

of some sort of guidance of fibres by Schwann cell tubes. After nerve transection, he demonstrated that the old pattern of reinnervation was not restored but regenerating fibres did return to old receptor sites. This would certainly seem to be a very plausible explanation for the results of the work presented here. The recovery of receptive field area after nerve crush was very good, as was the recovery after nerve-to-nerve suture, but the difficulty in mapping receptive field areas after nerve transection and repair may be due to a poorer recovery of receptor density. A better recovery of receptor density after injuries of the axonotmesis type probably results from the guidance of regenerating fibres to their original end-organs.

Diamond *et al.* (1976) said: "it appears that an interaction between the nerve and the target tissue controls the density of the endings, while the area of a terminal field is more determined by spatial relations". These spatial relations and 'body space' constrictions are seemingly lost by regenerating nerve and the less than complete recovery of receptive field area may well be due to the gaining of peroneal terrritory by the neighbouring sural nerve.

(c) Recovery in diabetes.

The recovery of the peroneal receptive field ratio in the diabetic nerve crush, nerve-to-nerve suture and nerve graft groups was poorer than their equivalent non-diabetic groups, although not significantly so ($p > 0.05$). Unlike the non-diabetic animals, the mean receptive field ratio of the diabetic muscle graft group was significantly smaller than any of the other groups ($p < 0.01$). The recovery of mean receptive field ratio after muscle grafting was significantly poorer in the diabetic rats than in the non-diabetic animals ($p < 0.01$).

The fact that the mean receptive field ratio of the control groups both approximate to unity has been discussed above. The mean receptive field ratio of the operated groups of diabetic rats was smaller than the equivalent in the non-diabetic rats in every group. This suggests that the recovery of receptive field area is poorer in diabetics, at 150 days, although in statistical tests no significant difference was found except between the muscle graft groups (see below). The reason for poorer recovery of sensation in diabetics is a matter for conjecture. The theories in existence that could explain the reduced capacity of peripheral nerve to regenerate in diabetes have been discussed in chapter 1.8. One theory involves poor signalling to the cell bodies of regenerating nerve fibres of nerve injury and this results in a decrease in the activation of certain enzymes in the cell body and a consequent decrease in the transport of the building blocks, required for regeneration, to the site of damage. One of the proposed signalling factors which stimulate the activation of ODC in the dorsal root ganglion is NGF (Kanje *et al.*, 1986) and Jakobsen *et al.* (1981) have reported poor retrograde transport of NGF to be a feature of STZ diabetes. Brown, Perry, Lunn, Gordon and Heumann (1991) reported that the ability of sensory nerve axons in a certain breed of mice to regenerate was poor. Wallerian degeneration is known to be slow in C57BL/Ola mice and it was suggested that the mechanism behind the poorer regeneration in these mice, as compared to C57BL/6J and BALB/c mice, in which degeneration is normal, was due to poor recruitment of macrophages to the site of injury. Macrophages are thought to initiate mitosis in Schwann cells (Beuche & Friede, 1984) and induce them to manufacture NGF (Heumann *et al.*, 1987). It may be that abnormal NGF levels and/or abnormal axonal transport of NGF is the common link between the poor regeneration seen in the sensory fibres of C57/Ola mice and STZ diabetic rats. Diamond *et al.* (1987) reported

that antibodies to NGF did not interfere with the regeneration of nociceptive axons in adult rats although they did prevent collateral sprouting. However, Brown *et al.* (1991) demonstrated that injections of NGF into saphenous nerve improved regeneration and they claim that "there is a degree of heterogeneity among sensory axons in their trophic requirements which is quite apart from the very clear difference now shown to exist between motor and sensory axons". Therefore, NGF levels in injured nerve may well affect regeneration of some types of sensory neurons. In STZ diabetes, it is the retrograde transport of NGF that has been reported (Jakobsen *et al.*, 1981) and this could account for the reduced induction of ODC in the cell body (Kanje *et al.*, 1986).

Importantly, Brown *et al.* (1991) demonstrated that the levels of NGF mRNA and NGF receptor mRNA were lower in the transected sciatic nerves of their C57BL/Ola mice and although the regeneration of sensory fibres was impaired, the motor axons regenerated quite well. In the work described in the next chapter the degree of recovery of motor function was investigated. Brown *et al.* (1991) also noticed that the regeneration of proprioceptive fibres in their C57BL/Ola mice was slow. Verge *et al.* (1989) produced evidence of muscle spindles being NGF responsive, however, Davies (1986) showed that during early development, proprioceptive fibres are not responsive to NGF. In Chapter 7, experiments on the recovery of proprioceptive reflexes are described.

CHAPTER 6

Assessment of Motor Recovery

6.1 Introduction.

6.1.1 Aims.

Fernand and Young (1951) noticed that there was no correlation between nerve fibre size and the amount of muscle tissue or numbers of muscle fibres reinnervated in the adult rabbit. Quite naturally, patients will feel that the return of muscle function is far more important than the recovery of nerve fibre size. The aims of this chapter are to describe the investigations in to the recovery of muscle function 150 days after different types of nerve injury and repair and to compare the recovery with similar repair groups of diabetic animals. Isometric twitch contractions of the soleus muscle were recorded from all groups of animals (described in chapter 2.5). Isometric tetanic tension was recorded from a parallel set of animals. This latter set of animals was also used in the reflex experiments of chapter 7.

6.1.2 Structure and Function of Skeletal Muscle (with special reference to rat soleus).

The types of extrafusal muscle fibre that compose mammalian skeletal muscle have been classified by numerous authors depending on structural and histochemical differences. Three basic types of fibre have been identified: slow oxidative (type I), fast glycolytic (type II) and intermediate (type III) (see Close, 1972 for review). The advantage of using the soleus muscle in experiments is that this muscle is almost entirely composed of the same fibre type. In fact, 80-90% of rat soleus muscle fibres are slow oxidative (type I), the remaining 10-20% are intermediate (type III) fibres (Close, 1972). Between 10 and 1000 muscle fibres, typically of the same

type and served by branches of the same motoneurone, form a motor unit. Motor units represent biological amplifiers whereby a few motoneurones can activate the generaton of large isometric forces (Burke, 1980). Usually, the muscle fibres of a single motor unit are widely distributed in a muscle. As in the grouping of muscle fibres, motor units have also been 'typed'. The motor unit types include: FF fast-twitch fatiguable, FR fast-twitch fatigue resistant, F(int) fast-twitch intermediate and S slow-twitch (Burke, 1980). Close (1967) found the rat soleus to consist of 30 motor units, of which 27 were slow-twitch and 3 were fast-twitch intermediate. The soleus muscle has a rich blood supply and a high mitochondrial content (Lewis & Ridge, 1982) which, in part, allow for the necessary fatigue resistance of this and other postural muscles.

Each muscle fibre contains bundles of thick (myosin) and thin (actin) filaments which are the contractile elements of the fibres. The filaments interdigitate and lie in parallel with each other. The length of the thick myosin filaments in rat leg muscles are 1.6 μm and the length of the thin actin filaments are 2.3 μm. One contractile unit is called a sarcomere and the length of a mammalian sarcomere at rest is approximately 2.8 μm (Lewis & Ridge, 1982). According to Close (1972), the optimum initial length of the rat soleus sarcomere for obtaining maximum twitch contraction was 2.84 μm at 35 ^{o}C. This is in agreement with findings of optimum length for recording maximum isometric twitch tension in whole muscle. Rack and Westbury (1969) found that the optimum length for producing isometric twitch tension in cat whole muscle was close to resting length and that a similar length was also optimum for producing tetanic tension. Close (1972) said that the relationship between the length and tension of the muscle being investigated is one of the most important conditions that must be established before performing experiments on that muscle and for twitch

contractions, the optimum resting length is greater than the optimum for tetanic contractions by 5-10%. Increasing the length of some muscles to more that 1.3 times their resting length can cause irreversible detrimental effects when measuring isometric tension.

Force in a muscle is generated by excitation-contraction coupling. An action potential across the sarcolemma spreads throughout the system of T-tubules of the muscle and causes a release of Ca^{2+} ions from the sarcoplasmic reticulum. In the resting state, the troponin-tropomyosin complex (which lies in the groove of the the actin helix) inhibits interaction between myofilaments of actin and myosin by preventing hydrolysis of ATP. When Ca^{2+} binds to troponin, the pre-existing inhibitory state is removed and actin can bind to myosin. Cross-bridges between actin and myosin are continually formed and broken while the muscle is in the 'active state', thereby bringing about shortening of the muscle. Relaxation of the muscle occurs when Ca^{2+} is removed from the troponin-tropomyosin complex and sequestered by the sarcoplasmic reticulum from which it was initially released.

6.1.3 Isometric v Isotonic Contraction.

Muscle contraction consumes energy. During an isometric contraction the length of the muscle does not change and, consequently, all the energy is used in generating tension in the muscle. This contrasts with an isotonic contraction during which the muscle shortens and external work is done to move a constant load. Isometric contractions are generally used in experiments on muscle because it is easier to produce an apparatus where the length of a muscle can be fixed between two points and the tension produced in that muscle measured, than it is to produce a situation where the muscle is working against a constant load. Also, the results from

isotonic recordings may be invalid because work is done and energy is used in overcoming frictional forces and/or overcoming the inertial load of adjacent inactive fibres (Lewis & Ridge, 1982). Lewis and Ridge (1982) said that the total tension developed in a muscle as a result of an isometric twitch contraction is the sum of passive and active tension. The passive tension is the tension in unstimulated muscle and this increases as the muscle is stretched. The active tension is the tension produced during contraction of the muscle and equals the total tension minus the passive tension.

6.1.4 Isometric Twitch v Tetanic Contraction.

The tension produced during an isometric contraction is dependent on the number of active motor units and also on the frequency of contraction of those motor units (Lippold, 1952). Different types of motor unit contribute differently to the total tension produced by a muscle. For example, the slow motor units of cat medial gastrocnemius make up 25% of the total motor unit population of that muscle but contribute only 5% to the total isometric force (Burke, 1980). The total tension developed during an isometric twitch contraction is much smaller than the tension produced during an isometric tetanic contraction. This is to do with the passive series elastic component of muscle which is found partly in the tendons and partly distributed along the muscle fibres (Desmedt & Hainaut, 1968). Supposing the tension during a twitch contraction were developed instantaneously, the force recorded at the tendon would rise slowly because of stretching of the elastic component. The duration of the twitch contraction is such that the developing tension never reaches values anywhere near those of tetanic contractions; in the latter, a continuous 'active state' is produced. The effect of the series elastic component and the gradual development of tension

during a contraction are the reasons why values of twitch tension are only a fraction of tetanic tension.

6.1.5 Measurement of Isometric Tension.

In the past, tension produced by muscle has been expressed as the tension produced per gram of muscle mass. This measurement is limiting because not all of the measured mass of a muscle is contractile tissue and the amount of tendon, connective tissue *etc*. may well vary from muscle to muscle. The tension produced in isometric contractions has also been described in terms of tension per unit cross-sectional area (Hartree & Hill, 1921; Close, 1969; Bàràny & Close, 1971). This measurement has been used because the difference in the tension developed by two muscles is independent of their lengths but is dependent on a difference in cross-sectional area. The mean cross-sectional area can be calculated as follows:

(1) $$A = V/l$$

where: A = mean cross-sectional area

V = volume of the muscle

l = length of the muscle

(2) $$V = M/D$$

where: M = mass of the muscle

D = density of the muscle

Inserting equation (2) into equation (1):

(3) $$A = M/Dl$$

Assuming that the density of muscle is similar for all muscles, the cross-sectional area is proportional to M/l. One of the disadvantages of using this method as an estimate of mean cross-sectional area is that l is a measure of muscle length and not muscle fibre length and this can lead to an over-estimation of the force generated by the muscle (Close, 1972). The assumption that density is similar for all muscles may be valid but density gives no indication of the amount of contractile tissue occupying a unit volume and myofilaments are present in higher densities in fast-twitch than slow-twitch muscle (Lewis & Ridge, 1982). In experiments on degeneration and regeneration such as those presented in this thesis, the density of contractile tissue is more likely to vary from muscle to muscle, as is the amount of contractile tissue per unit of muscle mass. It is because of the difficulties in finding a reproducible standard by which to compare the tension produced in different muscles that, in the work presented here, the maximum twitch and tetanic tensions developed in each soleus muscle were measured. The mass of the muscles was also determined to give an indication of the amount of contractile tissue present.

Previous authors have demonstrated that the tetanic tension produced in the soleus muscle of female rats is approximately two thirds of the tension produced in extensor digitorum longus (Close, 1969). Close suggested that the difference between the two muscles is probably due to extrinsic factors that influence activation and not to differences in the density of contractile material. However, Lewis & Ridge (1982) stated that myofilaments are present in higher densities in fast-twitch muscle fibres than in slow-twitch muscle fibres and consequently, the tension produced per unit cross-sectional area is some 50% higher in fast-twitch than slow-twitch muscle. Close (1967) determined the maximum isometric tetanic tension of motor units of rat skeletal muscles. He found the maximum isometric

tension of both fast and slow units was 4.7 gwt, whereas the value for the intermediate units was 5.85 gwt. This is in contrast to the findings of Burke (1967) who studied the tension produced by fast and slow motor units of cats. Of the 43 fast units studied, only 9 fast units developed tension less than 0.2 N, whereas all of the slow units developed tension less than 0.2 N.

6.1.6 Potentiation of Twitch Contractions.

The 'active state' of a muscle has been described as the capacity of the muscle to shorten or develop tension (Desmedt & Hainaut, 1968). The double-shock experiments of these authors demonstrated that the intensity of the 'active state' was not maximal after a single supramaximal stimulus and that a second stimulus (after a minimum separation interval) could augment the rate of development of tension. The tension produced during an isometric twitch can be potentiated using chemicals (e.g. caffeine, nitrate and zinc) but tetanic contractions are not increased in the same way (Desmedt & Hainaut, 1980). Desmedt and Hainaut (1980) suggested that the chemical potentiation of twitch tensions occurred because the duration of the 'active state' was increased, thus allowing greater time for stretching of the series elastic component. They also suggested that the intensity of contraction of the contractile elements was increased. Staircase potentiation of the isometric twitch occurs on stimulation of rested muscle at a rate of 0.5-3 Hz. Desmedt and Hainaut (1968) delivered supramaximal electric pulses to the human ulnar nerve at infrequent intervals. They noticed that after the first 10-20 twitches, the twitch tension decreased (negative staircase) but further stimulation caused an increase in the measured tension which reached a steady state value of 136% the initial twitch tension. They also noticed that the latter twitches had shorter contraction and half relaxation times. They argued, therefore, that staircase potentiation

occurred not because of an increase in the duration of the 'active state' but because of an increase in the intensity of contraction of the contractile elements. This increase in intensity of contraction could be explained by a decrease in the mechanical threshold for contraction such that a muscle action potential causes a greater release of Ca^{2+} than it would under normal conditions and/or the reuptake of Ca^{2+} by the sarcoplasmic reticulum could be increased.

6.1.7 Post-tetanic Potentiation.

It has been suggested that the concentration of released Ca^{2+} determines the relative peak twitch tension developed in a muscle (Close & Hoh, 1968). This theory has arisen partly as a consequence of the observation that after tetanizing a muscle, a single post-tetanic stimulus results in a twitch tension larger than the tension produced by a similar stimulus given before the tetanus. This effect is called post-tetanic potentiation and occurs in many mammalian limb muscles when they are stimulated either directly or indirectly (Nyström, 1968). Nyström (1968) suggested that an increase in twitch tension is possible if the muscle fibres are not fully activated during a pre-tetanic twitch contraction but in a post-tetanic twitch contraction, there is an increase in the number of parallel contractile elements that are active at any one time. This could occur if the number of active myofibrils and/or the number of active myofilaments is increased. Close and Hoh (1968) proposed that varying levels of Ca^{2+} 'activator' could explain post-tetanic potentiation. They said that if all the actin-myosin cross-bridges of a fibre contribute to the tension developed in that fibre, and the average rate of cycling of cross-bridges is limited by the amount of 'activator' present after the action potential, then it is possible for the average rate of cycling of cross-bridges to be increased if the amount

of 'activator' is also increased. Thus, if after a single action potential, the concentration of 'activator' (i.e. Ca^{2+}) is submaximal but after a second stimulus the amount 'activator' released is increased, this would result in a greater rate of turnover of cross-bridges. The tension produced in the muscle would, therefore, be increased on repetitive stimulation. In addition, the increase in concentration of Ca^{2+} would explain the finding that on repetitive stimulation, the duration of each twitch contraction is increased. The duration of the muscle twitch would be increased because it would take longer for the concentration of Ca^{2+} to fall below subthreshold levels and bring about relaxation of the muscle. Nyström (1968) found there was a post-tetanic potentiation of about 50% in the soleus muscle of kittens but there was practically no potentiation in adult cats.

6.1.8 Duration of Muscle Contraction.

The total shortening velocity of a muscle fibre is the sum of the velocities of its sarcomeres. The duration of muscle contraction is important when investigating muscle denervation and reinnervation because the contraction time of a muscle depends on the types of fibre/motor unit present and these may change after reinnervation (see below). The isometric twitch contraction of fast-twitch muscle is approximately three times briefer than that of slow-twitch muscle (Lewis & Ridge, 1982).

Bàràny (1967) demonstrated that actomyosin ATP-ase activity was related to the shortening velocity of muscle and the shortening velocity is probably limited by the rate at which cross-bridges are made and broken. Rate-limiting of a muscle twitch may be produced by:

(1) Controlled release and uptake of Ca^{2+} by the sarcoplasmic reticulum. The rate of uptake of Ca^{2+} by fast muscle fragments was found to be 4-11 times greater than that of slow muscle fragments at room

temperature and fast muscles were found to contain twice as much sarcoplasmic reticulum as slow muscles (Fiehn & Peter, 1971). Hence, it is feasible that the release and uptake of Ca^{2+} from the sarcoplasmic reticulum is quicker in fast muscle than slow muscle. Consequently, threshold levels of Ca^{2+} are more rapidly attained in fast muscles as opposed to slow muscles to bring about contraction and relaxation.

(2) An 'intrinsic' control over the rate of muscle contraction and relaxation for a given amount of Ca^{2+} released into the sarcoplasm e.g. the intrinsic speed of muscle shortening is determined by the rate of splitting of actomyosin by ATP. Close (1965) showed that there was an inverse relationship between duration of muscle twitch and maximum speed of shortening of contractile material and myosin ATP-ase activity. Close (1972) stated that "this is good evidence that the maximal rate at which thick and thin filaments slide past one another during contraction is limited by the rate of hydrolysis of ATP by myosin at individual enzyme sites".

6.1.9 Recovery of Muscle Function.

Gutmann (1942) investigated the recovery of function of muscle after nerve crush and nerve transection and repair by epineurial suture of the peroneal nerve in rabbits. Stimulation of the nerve produced contraction of muscle at 18-20 days after nerve crush and full return of the toe-spreading reflex occurred 8 days later. The return of muscle contraction in response to nerve stimulation was slower after nerve-to-nerve suture and the reappearance of the toe-spreading reflex was delayed even further. In fact, the toe-spreading reflex increased only gradually after nerve-to-nerve suture and never returned to normal. Berry, Grundfest and Hinsey (1944) demonstrated that after transection and suture of the sciatic nerve in cats, stimulation of the regenerating nerve could produce muscle twitches in the gastrocnemius as early as 23 days but animals did not begin to use the muscle until 50 or 60 days. These authors reported that in nerve crush studies they had observed almost complete restoration of the use of the affected limb but after nerve-to-nerve suture, cats never regained normal use. It is possible that the reduction in the force produced by muscle that has been reinnervated after an injury of the neurotmesis type is due to a decrease in the number of axons newly innervating that muscle. However, Luff, Hatcher and Torkko (1988) demonstrated that reinnervation of partially denervated hindlimbs of cats resulted in an increase in the size of motor units and that full recovery of muscle force could be produced with only 5% of motor axons intact. The reduction in the force generated by a reinnervated muscle after nerve transection and suture could also be

explained by atrophy of the muscle. Gutmann (1942) found that the normal weight of the tibialis anterior muscle in his rabbits was nearly regained 12 weeks after return of reflex function following a nerve crush injury. Finkelstein, Dooley and Luff (1993) found that 8 weeks after nerve-to-nerve suture of the nerve to the medial gastrocnemius muscle in rats, the muscle weights of the medial gastrocnemius were 25% less than controls.

Finkelstein *et al.* (1993) investigated the reinnervation of rat medial gastrocnemius after different periods of denervation. They found a progressive decline in the force recovered by the muscle after increasing periods of denervation and claimed that this was because the muscle loses the ability to recover and not because axons lose the ability to reinnervate the muscle. These authors cross-innervated the nerve to the medial gastrocnemius with the nerve to the lateral gastrocnemius and the nerve to soleus and compared the recovery of force produced in the reinnervated muscle with a group of animals where the medial gastrocnemius had been reinnervated with its proper nerve. They found that the number of available axons for regeneration had no effect on the recovery of force of the muscle and concluded that poorer recovery of muscle seen after longer periods of denervation was due to the inability of the muscle itself, to recover. However, they did not actually count the number of motor axons reinnervating the muscle. In their conclusion, they assumed that if recovery of muscle function is dependent on reinnervation, there should have been a difference in the recovery of denervated muscle when it was provided with fewer regenerating axons as compared to that provided with additional regenerating axons . But increased numbers of regenerating axons does not necessarily mean more functional connections will be made and an increase in the number of axons reinnervating a muscle does not necessarily mean better recovery of function (Luff *et al.,* 1988). These

authors also found that after longer periods of denervation (21 and 56 days), the size of muscle fibres that were cross-innervated by the nerve to the lateral gastrocnemius and the nerve to the soleus muscles were larger than the fibres that were reinnervated by their proper nerve fibres. They suggested that this was indicative that nerve axons lose the ability to maintain large muscle fibres. From the results of their work, Finkelstein *et al.* (1993) claimed that denervated muscle was more difficult to reinnervate. This finding is important to the experiments presented in this thesis when comparing nerve and muscle graft groups with nerve-to-nerve suture groups, because there is likely to be a longer delay in the arrival of regenerating axons at muscle after nerve injuries with two suture lines than injuries with one (Gutmann *et al.*, 1942). Finkelstein *et al.* (1993) proposed two different hypotheses to explain the poorer recovery of muscle after longer periods of denervation. The first involves the release of a 'factor' from denervated muscle that encourages axonal sprouting and that this 'factor' is produced in smaller amounts as the time of denervation progresses. The alternative hypothesis is that nerve is prevented from reinnervating muscle by a physical barrier such as that provided by fibrosis. Virtanen, Tolonen, Savolainen and Takala (1992) showed that denervation atrophy is associated with an increase in the rate of muscular collagen biosynthesis. However, they also showed that the rate of collagen synthesis decreases during reinnervation which suggests that innervation is a powerful regulator of muscular collagen biosynthesis. In order for the events descibed in the second hypothesis to be the limiting factor in muscle reinnervation, a large amount of collagen would have to be produced over relatively short periods of time to have any significant effect on muscle reinnervation.

6.1.10 Redistribution of Motor Units.

During neuromuscular development, the polyneuronal innervation that exists in very young mammals disappears as a result of synapse elimination. Should nerve injury occur in the adult, nerve regeneration transitorily results in a period of polyneuronal innervation that is rapidly followed by a period of 'organized elimination' (Ribchester, 1993). The result of 'organized elimination' is that a motor unit organization is produced that is similar but not identical to that which was present before denervation of the muscle.

The size and distribution of motor units is known to change after denervation and subsequent reinnervation. Bagust and Lewis (1974) demonstrated that denervation and self-reinnervation of the soleus muscle in cats causes a change in the distribution of values of motor unit tension. In the normal animal the tensions produced by motor units of the soleus muscle were normally distributed, however after reinnervation, the values of motor unit tension were skewed towards larger values. The nerves had been allowed to regenerate for 207 days so that there was little chance that the recording of larger tensions was the result of polyneuronal innervation.

6.1.11 Redistribution of Muscle Fibres.

In reinnervated muscle, it has been observed that there is a change in the distribution of the muscle fibres which make up the motor units. In normal muscle, the fibres of a single motor unit are widely distributed in that muscle. Kugelberg, Edström and Abbruzzese (1970) found that in reinnervated muscle, the number of muscle fibres per motor unit was within normal limits but the unit territories were reduced and, therefore, the packing density of the fibres was increased. The theory behind the change in the distribution of muscle fibres belonging to one motor unit is that

regenerating axon sprouts are more likely to make contact and innervate adjacent denervated fibres than distant ones. Thus, branches of a single motoneurone will tend to reinnervate muscle fibres situated more closely.

6.1.12 Grouping of Fibre Types.

The tendency for axons to reinnervate muscle fibres that are more closely situated to each other explains the finding that similar types of muscle fibre are grouped after reinnervation of that muscle. This is because different types of motoneurone, tend to innervate a single type of muscle fibre (see below) and hence, motor units tend to be composed of one fibre type (Close, 1972). If regenerating nerve fibres transform muscle fibres to the type of its own motor unit, it is not surprising that grouping of fibre types occurs. Nemeth, Cope, Kushner and Nemeth (1993), however, found that type grouping was not predominant in the self-reinnervated medial gastrocnemius muscle of the cat.

6.1.13 Neural Influence on Muscle Fibre Typing (evidence from cross-innervation experiments).

Buller, Eccles and Eccles (1960a; 1960b) proposed that the primary influence on the speed of muscle contraction is neurogenic. Buller *et al.* (1960a) found that transection of the spinal cord in new-born kittens caused a failure in the full differentiation of the slow soleus and crureus muscles. In a second paper, Buller *et al.* (1960b) demonstrated that cross-union of fast and slow motor nerves caused an increase in the speed of contraction of slow muscle and a decrease in the speed of contraction of fast muscle and that the reason for this was the influence of motoneurone type on muscle fibre type. They also demonstrated that this neural influence operated throughout life. Further evidence has been provided by Close and Hoh

(1968) who found that cross-union of motor nerves to fast and slow muscles in the rat had reciprocal effects on post-tetanic potentiation seen in these muscles. Regeneration of fast motor nerve into the slow soleus muscle resulted in the appearance of post-tetanic potentiation that is normally absent in adult slow muscle. Regeneration of slow motor nerve into the fast extensor digitorum longus muscle resulted in the disappearance of post-tetanic potentiation in that muscle. Buller *et al.* (1960b) suggested that the neural influence on muscle fibre type is exerted in some way by the different frequencies of discharge of fast and slow motoneurones. An alternative explanation is that the neural influence on muscle fibre type is neurotrophic in nature. Fischbach and Robbins (1969) investigated the changes in rat soleus muscle after immobilization of the knee and ankle joint. They found that the usual tonic firing of the motoneurones serving the soleus became more phasic in nature (i.e. more similar to motoneurones serving fast muscle). They also noticed that the soleus contraction time and tetanus/twitch ratio were decreased. They argued that because the muscle remained innervated and still underwent changes suggestive of a change in fibre type, the neural influence on muscle fibre type was unlikely to be neurotrophic unless the neurotrophic influence were partially dependent on neuronal activity.

6.1.14 Specificity of Muscle Reinnervation.

In experiments where nerves are transected and then sutured or grafted, any neural influence of regenerated nerve fibres on muscle fibre type and muscle physiology may not be as obvious as after cross-union experiments. This is because the extent of cross-innervation will vary. The degree to which cross-innervation occurs may be purely random or it may be affected by specificity of axonal regeneration such that fast

motoneurones preferentially reinnervate fast-twitch muscle fibres. Miledi and Stefani (1969) argued that the reinnervation of rat soleus muscle after transection and regeneration of the sciatic nerve was non-specific. They showed that the histological and physiological properties of the reinnervated muscle (including sensitivity to acetylcholine and isometric twitch contraction time) changed suggesting that there was an increased proportion of fast fibres in the reinnervated muscle. The sensitivity of extensor digitorum longus to acetylcholine was unaltered. Miledi and Stefani (1969) proposed that reinnervation of muscles was random and that the preponderance of fast motoneurones in the sciatic nerve (and possibly a greater rate of regeneration of fast motoneurones) could explain the changes in the soleus muscle after reinnervation. More recently, Lewis and Chamberlain (1993) published their work on the reinnervation of rat soleus and plantaris muscles after transection and reunion of the medial popliteal nerve (which is common to both of these muscles). They found that the differences in twitch contraction time between the slow soleus muscle and fast plantaris muscle persisted but were reduced after reinnervation. Differences in the histological types of muscle fibres between muscles were also found to be very different after nerve regeneration. Although after regeneration the soleus muscle contained more type II (presumed fast fibres) than control muscle, the proportion of type I fibres (presumed slow fibres) present was still very much greater than that in the plantaris muscle after operation and that proportion calculated for a model of random reinnervation by nerve fibres. Similar work by Gillespie, Gordon and Murphy (1986; 1987) showed different results from Lewis and Chamberlain (1993) in that reinnervated soleus muscle contained far more type I fibres (70% as opposed to 33%). Gillespie *et al*. (1986) showed no difference in the speed of contraction of reinnervated soleus as compared to control values,

whereas, Lewis and Chamberlain (1993) demonstrated a considerable reduction in the speed of contraction of soleus, although the difference between soleus and plantaris was maintained. Both Gillespie *et al.* (1986) and Lewis and Chamberlain (1993) agreed that although reinnervated fast and slow muscles did maintain different physiological and histochemical differences, there was no proof of selective reinnervation. Gillespie *et al.* (1986) suggested that the greater proportion of slow contracting fibres found in soleus, than would be expected by random reinnervation, might be due to resistance of soleus fibres to conversion. Lewis and Chamberlain (1993) disagreed with this theory and proposed that the higher proportion of slow fibres maintained in soleus after reinnervation be explained by motoneurones being influenced by the muscle that they innervate. The properties of fast and slow motoneurones differ in that fast motoneurones have much higher frequencies of discharge than slow motoneurones and this may be explained by the longer hyper-polarizations of slow motoneurones (Buller *et al.* (1960a). Kuno, Miyata and Munoz-Martinez (1974) demonstrated 'dedifferentiation' of fast and slow motoneurones after axotomy of the medial gastrocnemius and soleus nerves in cats. Axotomy produced changes in the properties of both fast and slow motoneurones. The authors suggested that the 'dedifferentiated' neurones may be 'redifferentiated' after reinnervation of muscle (Kuno, Miyata and Muno-Martinez (1974).

The theory proposed by Lewis and Chamberlain to explain the larger than expected proportion of slow muscle fibres in reinnervated soleus muscle involves the production of a trophic substance that is required to maintain motoneurones after reinnervation. If fast muscle fibres produce more trophic substance than slow muscle fibres, the muscle fibres of soleus would produce less trophic substance and consequently, fewer fast

motoneurones reinnervating soleus would have their properties restored to normal. The fast motoneurones whose properties had been changed as a result of axotomy to properties more similar to slow motoneurones, might not be able to convert slow muscle fibres to fast fibres as described by Buller *et al.* (1960b).

6.2 Materials and Methods.

6.2.1 Preparation of Experimental Animals.

150 days after the initial operations on the sciatic nerve, described in chapter 2.6, each rat was anaesthetized (chapter 2.3) and kept warm on an electrically heated blanket. The left leg was shaved using electrically operated clippers and the sciatic nerve was exposed (chapter 2.4).

The sciatic nerve was viewed, and all dissection carried out, under an operating microscope (Weck Fibermatic 0902A1, Long Island City, New York). The nerve was freed from the surrounding muscle bed and followed distally to its branching point into tibial and peroneal nerves. The peroneal and sural nerves were divided immediately distal to the point of junction with the sciatic nerve.

6.2.2 Exposure of the Soleus Muscle.

An incision was made from the ankle of the left hindlimb to the popliteal fossa. The skin was freed from the muscle below and bleeding was stopped using a Codman Malis bipolar coagulator (Codman and Shurtleff Inc., Randolph, Massachussettes, U.S.A.). A 5/0 Flexon stainless steel multistrand suture (Davis and Geck American Cyanamid Co., U.S.A.) was passed through the tendo calcaneus and tied. The stainless steel suture was used to connect the soleus muscle to the tension transducer. The reason for choosing a stainless steel suture was that the aims of the experiments described here were to measure the tension produced in the muscle, therefore, it was necessary that the suture connecting the muscle

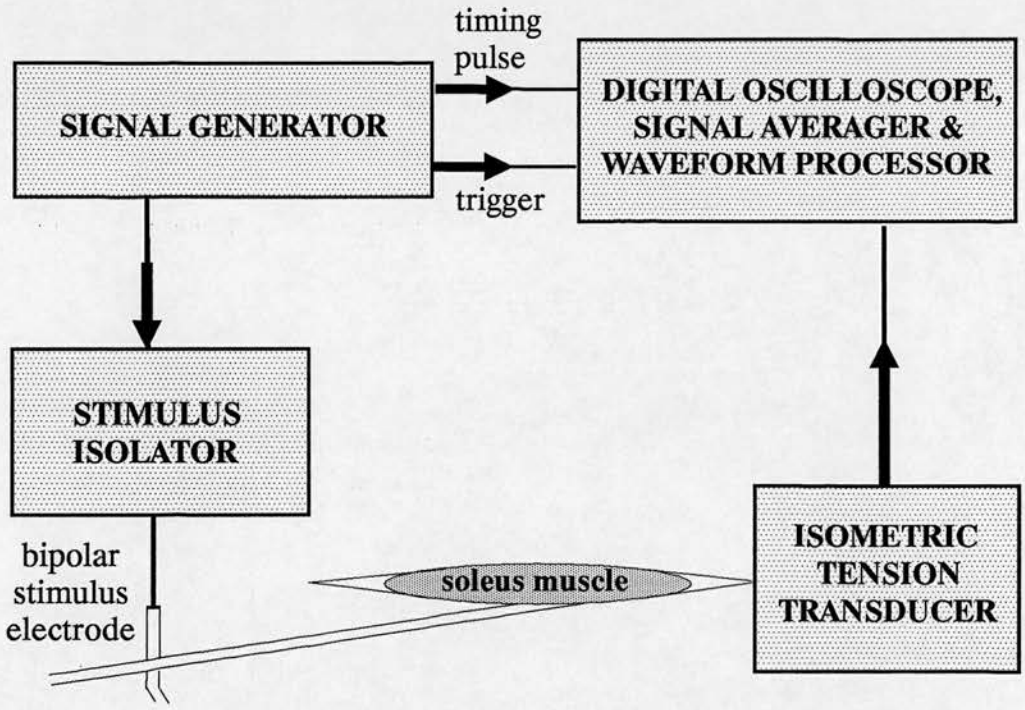
to the transducer was inextensible. Using a scalpel, the tendon was divided distal to the wire tie and pulled away from the limb. This freed the muscle bulk from the surrounding structures. The gastrocnemius was dissected away from the soleus muscle lying deep to it and was removed from the preparation. Cellophane was placed over the soleus in an attempt to minimize evaporation and hence cooling of the muscle. Large fluctuations in temperature are known to cause variations in the maximum force of muscular contraction (Close, 1972).

6.2.3 Mechanical Apparatus.

The animal was moved from the operating table to an earthed table inside a Faraday cage and laid on its back. The tibia and femur were held rigidly by two clamps so that the tibia was held almost perpendicular to the table. The clamps were fixed to a Vernier which moved in a horizontal plane and was rigidly supported by a metal post. This enabled the insertion of soleus to be moved in small increments, towards or away from the tension transducer. The wire attached to the tendon of the soleus muscle was tied to the beam of an isometric tension transducer (Harvard Apparatus #52-9503, Edenbridge, U.K.). By moving the Vernier, the muscle could be stretched to a constant length at the commencement of each twitch. Changing the length and, therefore the tension in the muscle, caused a DC deflection of a trace which was displayed on an oscilloscope screen (see below for electrophysiological apparatus). The point at which there was minimal tension in the muscle was recognized when shortening the length of the muscle, by moving the Vernier, no longer caused a deflection in the trace. By this method, the tension in each soleus muscle used in the experiments described here, was set at 0 N at the commencement of each experiment. It was important to standardize the length of the muscle

because the force of contraction of a skeletal muscle varies as the initial length (Close, 1972). This is to do with optimal positioning of the contractile filaments of the muscle, which is not achieved if the muscle is stretched too far.

Figure 6.1 Diagram of the electrophysiological apparatus used for measuring twitch tension produced in the soleus muscle



6.2.4 Recording of Twitch Tension.

A diagram of the electrophysiological setup is shown in figure 6.1. The soleus muscle was attached to the beam of the isometric tension transducer #52-9503 (Harvard Instruments, Edenbridge, U.K.) with an inextensible stainless steel suture (see above). A bipolar, low impedance stimulating electrode was placed under the sciatic nerve, proximal to the repair site,

and a silver wire ground electrode was inserted into the gastrocnemius muscle. The table, Faraday cage and preparation were earthed via a common earth, which ultimately passed to ground by way of 'mains earth'.

The stimulating electrode was connected to a battery-powered Dagan 9250 stimulus isolator unit (Dagan Corporation, Minneapolis, U.S.A.) which was driven by a Dagan 9200 Omni pulse stimulator (Dagan Corporation, Minneapolis, U.S.A.). The stimulator was programmed to produce an output TTL timing pulse to the a y-input of a multichannel Gould 7074 Digital Storage Oscilloscope and Gould 704 waveform processor (Gould Electronics Ltd, Ilford, U.K.) synchronized with the delivery of the stimulus. The stimulus was set to have a delay of 2 ms after triggering and duration of 500 μs and was delivered at a frequency of 1 Hz. By using the digital TTL output from the stimulator to drive the stimulus isolator, a square wave analogue output was produced whose amplitude could be varied between 10 and 100 V. The unfiltered output from the tension transducer was amplified and sent to a y-input of the oscilloscope. The amplitude of the stimulus was incremented until the display of twitch tension on a channel of the oscilloscope reached a maximum. From preliminary experiments it was found that isometric tensions could vary quite considerably, therefore, using the signal averaging facility of the waveform processor, an average of 8 traces was recorded. Also using the waveform processor, various measurements were made from the averaged trace, using the display of the timing pulse as a reference trace for the stimulus. The peak twitch and time to peak twitch were measured. Although it has been found that the end-point of contraction is sometimes difficult to define (Hems, 1993), particularly after nerve repair when the twitch is drawn out, the duration of the twitch was measured.The time to 50% relaxation was also measured as

was the time to 50% of the peak value. The area under the curve was calculated using the waveform processor facility associated with the oscilloscope and this number was divided by the duration of the twitch to give a value called the time-tension index. The reasons for recording each of these variables are discussed in the results section of this chapter.

The stimulating electrode was moved to be in contact with the belly of the soleus muscle and the Omni pulse stimulator was set to deliver a stimulus which was incremented until the twitch induced in the soleus muscle reached a maximum. 8 traces were averaged as before and the same variables were measured.

6.2.5 Calibration of the Tension Transducer.

The isometric tension transducer (Harvard Instruments #52-9503, Edenbridge, U.K.) was clamped so that its beam was parallel to the table. Small weights in the range 2-140 gwt were suspended from the beam. The DC output of the tension transducer was connected to a channel on the oscilloscope. The deflection on the oscilloscope (in volts) was measured for each of 16 weights and a graph was plotted of mass x acceleration due to gravity (i.e weight/ N) against oscilloscope deflection/ V. Using Slidewrite software (Advanced Graphics Software inc., California, U.S.A.), the best fit straight line was drawn through the points and the equation of the line was calculated. The calibration curve is displayed in appendix 14.

6.2.6 Recording of Tetanic Tension.

Tetanic tension was recorded in the soleus muscle in different animals from those used in the experiments to record twitch tension. The animals used for recording tetanic tension were also used for the reflex tension experiments of chapter 7. It seemed more appropriate to discuss the results

from the tetanic tension experiments with those from the twitch tension experiments and for this reason, the description of the former wil be included in this chapter. The animals were anaesthetized and the soleus muscle was exposed exactly as described above. The animals were arranged on a table inside a Faraday cage in the same way as described in chapter 6.2.3. As in the experiments for recording twitch tension, the tension in the soleus muscle was set at 0 N at the commencement of each experiment. A bipolar low impedance electrode was placed under the sciatic nerve proximal to the site of nerve injury and repair and was connected to a battery-powered Dagan 9250 stimulus isolator (Dagan Corporation, Minneapolis, U.S.A.). The apparatus used for producing tetanic tensions in the soleus muscle of rats was not dissimilar to that described above. The Omnipulse 9200 was programmable to deliver trains of square wave impulses of which stimulus duration, voltage and frequency could be pre-set. The final analogue output was delivered from the battery powered stimulus isolator which was driven through an optical connector by the Omnipulse 9200. The amplitude of the stimulus was incremented until the tension produced in the muscle increased no further. The tension produced in the muscle as a result of the stimulus was displayed on the oscilloscope screen in the same way as described in chapter 6.2.4 and the waveform processing facility was used to measure the peak tension displayed on the oscilloscope screen.

6.2.7 Weighing of the Soleus Muscle.

The right soleus was exposed as in 6.2.2 and the muscle was removed from its proximal attachments using a scalpel. The left soleus was also cut from its origins. The muscles from both sides were 'weighed' using an Oertling HC22 balance (Fisons Scientific Equipment, Loughborough, U.K.).

6.3 Results.

6.3.1 Interpretation of the Isometric Myogram.

A typical isometric myogram is shown in figure 6.2. As previously described in chapter 6.2.4, data were collected from different points on the isometric myogram for statistical analysis. These variables included:

(a) Maximum isometric twitch tension (*F*). This is the maximal tension developed in a muscle as a result of stimulation by applying a single supramaximal stimulus. Maximum isometric twitch tension is a measure of the maximal force that can be produced by a muscle and "is the most fundamental expression of the mechanical response" (Hartree & Hill, 1921).

Hartree and Hill (1921) measured the heat produced in muscle that had been stimulated for different durations and at different temperatures. They found that the heat produced in the muscle per unit length divided by the tension produced in the muscle was independent of temperature for very short stimuli i.e.

$$Q/\,lF$$

is constant for short stimuli

where: Q = the heat produced in the muscle/ J or Nm

F = tension produced in the muscle/ N

l = length of the muscle/ m

This relationship is not surprising if the dimensions of (a)*Q* and (b)*IF* are considered:

(a) Heat is energy and is produced as a result of doing work. Work is the product of force and distance. The dimensions of force are:

$$MLT^{-2}$$

and the dimensions of distance are:

$$L$$

Therefore, the dimensions of heat are:

$$MLT^{-2} \times L = ML^2T^{-2}$$

(b) The dimensions of the product of force and length are:

$$ML^2T^{-2}$$

Therefore, considering the dimensions of *Q* and *IF* are the same, *Q*/ *IF* is dimensionless and it is not surprising that *Q*/ *IF* is constant for short stimuli. In the work described in this thesis, the duration of stimulus used was 50 μs and, by the standards of Hartree and Hill (1921), this is very short. The importance of measuring the maximium tension produced in the muscle is that it is not only indicative of the maximum force produced by the muscle but is also an indicator of the heat produced in the muscle per unit length (for short stimuli).

(b) Time to peak tension (*t*). This time includes the conduction time along the nerve, the time for neuromuscular transmission and the time for the passage of the action potential to spread via the muscle membranes and cause maximal contraction.

(c) Time to half peak tension ($t_{1/2}$). The reason for recording the time to half peak tension is that this value gives an indication of the shape of the curve of the isometric myogram before peak tension is reached. Supposing

a situation occurs where the times to peak tension of two different traces, *x* and *y*, were equal but the time to half peak of trace *y* was slower than that of trace *x*. Such a situation would be indicative of a slower initiation of contraction in muscle *y,* followed by a more rapid spread of contraction through the muscle, compared to muscle *x*. The slower initiation of contracton could be because of slower conduction of the nervous tissue or a delay in neuromuscular transmission.

(d) Time to half relaxation ($r_{1/2}$). Previous authors (Hems 1993, Myles, 1990) recorded the time from the start of the stimulus to the time on the downslope at which the tension of the muscle had relaxed to half of its maximal value. Considering that this measure of time includes the time taken to reach peak tension (see (b) above), it was decided that a better measurement to make was the time taken for the muscle to relax to half its maximal value, measured from the time at which maximal twitch tension had been attained. Such a measure is preferable because it gives a true indication of time taken for relaxation of the muscle. Using the variable measured by the aforementioned authors, it is foreseeable that different 'times to half relaxation' could be measured from two different muscles when the rate of relaxation of the two muscles was, in fact, equal.

(e) Duration of contraction (t_d). This is the time for completion of the muscle twitch from the delivery of the stimulus. Hems (1993) found that this time was very variable because of the "slow tailing-off of the contraction" and consequently, when measuring the area under the isometric myogram (see below), he used the time from delivery of the stimulus to the time for the tension developed by the muscle to relax to half of its maximum value. If tables 6.27 and 6.28 (which show the mean, standard deviation, standard

error of the mean and coefficient of variation for the time to half relaxation for non-diabetic and diabetic animals, respectively) are compared to the values of duration of contraction (tables 6.29 and 6.30 for non-diabetic and diabetic animals, respectively), it can be seen that there was greater variation in the time to half relaxation than total duration.

(f) Time-tension integral (*TT*). The area under the curve of the isometric myogram is called the time-tension integral. Myles (1990) and Hems (1993) used this measure as an indicator of the heat produced in the muscle (and thus work done during contraction of the muscle). In fact, the time-tension integral is not proportional to the heat produced during contraction of a muscle when the muscle is stimulated by brief stimuli. In their search to find a mechanical response that was proportional to heat production, Hartree and Hill (1921) investigated the relationship between heat production and the time-tension integral. They found that the integral was not proportional to heat production for short stimuli. This finding is very likely to be because the rate of heat production is not constant during a muscle twitch. The utilization of ATP during a muscle twitch is likely to be maximal during contraction when cross bridges between actin and myosin are being broken and re-formed. During relaxation, however, part of the work is done by passive extension and consequently, requires less ATP. In different muscles, the time taken for the muscle to relax during a simple isometric twitch may vary considerably (and did in the work of the experiments presented here; see tables 6.27 and 6.28). Such differences in contraction/relaxation times between one muscle and another means that the time-tension integral is highly unlikely to be proportional to heat production (and ATP consumption). In fact, a closer relationship between heat produced and a mechanical response of the muscle is that between heat production and

maximum isometric tension (see (a) above). Hartree and Hill (1921) found that the heat production in a time interval is proportional to tension multiplied by the time of contraction when the muscle is in a steady state of contraction. This finding fits the arguments above because during the steady state of contraction, the rate of doing work and ATP consumption is likely to be constant. Importantly, however, the time-tension integral is an indicator of another mechanical property of the muscle:

$$TT = k \int_{t0}^{td} F.dt$$

where: k = constant

F = tension/ N

t_d = time at the end of the muscle twitch/ s

t_0 = time at the beginning of the muscle twitch/ s

The time-tension integral will have the dimentsions of force and time.

The dimensions of force are:

$$MLT^{-2}$$

The dimensions of time are:

$$T$$

The dimensions of the time-tension integral are therefore:

$$MLT^{-2} \times T = MLT^{-1}$$

These dimensions are also the dimensions of momentum:

momentum = mass × velocity

The dimensions of momentum are:

$$M \times LT^{-1} = MLT^{-1}$$

and the S.I. units would be kg m s^{-1}

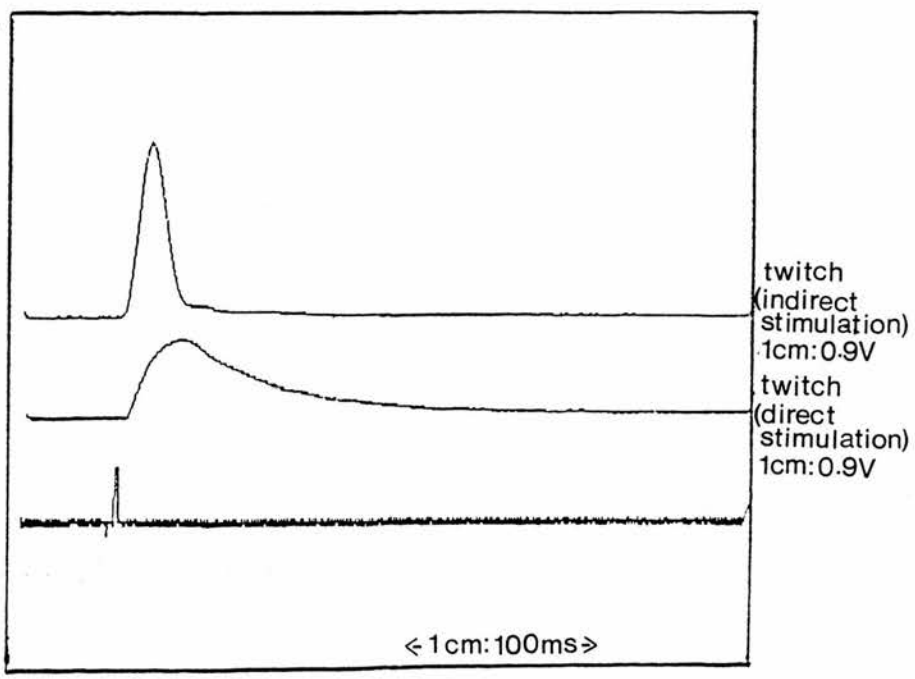
Hartree and Hill (1921) explained that the momentum produced in a large mass hanging at rest from a vertical string attached to a muscle which is stimulated, is proportional to the time-tension integral. The time-tension

integral, therefore, provides information about the ability of the muscle to move heavy masses and is an important mechanical property of the muscle.

(g) Time-tension index (*TTI*). By dividing the time-tension integral by the duration of the contraction the time-tension index is produced. This value represents an average of the tension produced in the muscle during the muscle twitch. Hems (1993) measured the area under the curve of the isometric myogram until the tension fell to half of its maximal value and divided this by the time when the tension in the muscle had relaxed to half of its maximal value (see (c) above). He claimed that this represented an 'average' tension for the contraction. In fact, his 'time-tension index' represented an average of the tension produced up until the time when the tension in the muscle had relaxed to half of its maximal value and, consequently, would be larger in magnitude than the average tension for the whole contraction.

As described in chapter 6.2.4, an isometric myogram was produced by direct stimulation of the muscle. Data were accumulated from similar points on the isometric myogram when the muscle had been stimulated directly. The reason for obtaining data by this method was to observe the function of the muscle, after nerve injury and regeneration, with a view to distinguishing the difference between recovery of muscle function and the combined recovery of function of nerve and muscle.

Figure 6.2 An isometric myogram recorded from the isometric twitch of the soleus muscle of a rat in the non-diabetic control group when the muscle was stimulated directly and indirectly



6.3.2 Results from Isometric Twitch Experiments.

Each of the control group, the nerve graft group, the muscle graft group and the diabetic muscle graft group contained 5 animals; the nerve crush group and the diabetic nerve graft groups each contained 7 animals; the diabetic control group and the diabetic nerve-to-nerve suture groups both contained 8 animals while the diabetic nerve crush group contained 9 animals and the nerve-to-nerve suture group contained 10 animals. Where the values of maximum isometric twitch tension *etc.* were recorded after stimulation of the sciatic nerve, they are referred to in the text as having resulted from indirect stimulation, and where the values were recorded after stimulation of the muscle membrane directly, they are referred to as having resulted from direct stimulation. Appendix 15 and appendix 16 show the values of maximum isometric twitch tension developed in the soleus muscle, the time to peak tension, the time to half peak tension, the time to half relaxation, the duration of the contraction, the time-tension integral and the time-tension index of each rat after indirect and direct stimulation, respectively. Parametric statistics were applied to the data as described in chapter 4.3.1.

6.3.3 Results from the Measurement of Maximum Isometric Twitch Tension (indirect stimulation).

The values of the mean and the coefficient of variation of maximum isometric twitch tension developed by the soleus muscle of the non-diabetic rats are shown in table 6.1. The equivalent values for the diabetic animals are shown in table 6.2. Construction of normal and half-normal plots showed that the values of maximum isometric twitch tension were normally

distributed, with no outliers. The residuals of maximum isometric twitch tension plotted against the fitted values of the mean for each group showed that each group had a similar variance.

Table 6.1 Values of the mean, standard deviation, standard error of the mean and coefficient of variation of the maximum isometric twitch tension of the soleus muscle (indirect stimulation) in non-diabetic animals

RAT GROUP	n	m /N	SD	SEM	c.v.
C	5	0.48	0.15	0.07	30.97
CR	7	0.32	0.13	0.05	39.83
NN	10	0.11	0.04	0.01	38.89
NG	5	0.15	0.12	0.05	75.49
MG	5	0.16	0.15	0.07	96.55

It can be seen from a comparison of tables 6.1 and 6.2 that the mean twitch tension produced by the non-diabetic control group was almost twice that of the diabetic control group. The mean tension produced by the soleus muscle tended to be greater in all of the non-diabetic operated groups as compared to their equivalent diabetic group. However, the biggest difference between non-diabetic and diabetic animals was seen in the control and crush groups.

Table 6.2 Values of the mean, standard deviation, standard error of the mean and coefficient of variation of the maximum isometric twitch tension of the soleus muscle (direct stimulation) in diabetic animals

RAT GROUP	n	m /N	SD	SEM	c.v.
DC	8	0.242	0.126	0.044	51.94
DCR	9	0.190	0.112	0.037	59.16
DNN	8	0.120	0.056	0.020	46.91
DNG	7	0.128	0.062	0.024	48.99
DMG	5	0.079	0.043	0.019	54.57

The duration of the experiments on the soleus muscle of the left limb was such that it was considered inadvisable to perform similar experiments on the right limb with the intention of using these values as controls. This is because the temperature of the soleus muscle fell throughout the course of each experiment and temperature is known to affect the development of maximum twitch tension (see chapter 6.4.1). In addition, compensation hypertrophy of the soleus muscle of the unoperated limb could not be discounted and to use values of tension recorded from these muscles as controls may well have been inappropriate. If the mean peak twitch tension that was obtained from each of the non-diabetic operated groups is expressed as a percentage of the mean of the non-diabetic control group, the percentage recovery of control twitch tension developed by the the different groups can be calculated. The percentage recovery of control twitch tension of the nerve crush group was 67.57%. The percentage recovery of peak twitch tension of the nerve-to-nerve suture group was 23.49%, and the equivalent values for nerve graft and muscle graft groups were 32.02% and 32.85%, respectively. The percentage recovery of mean peak tension of the non-diabetic nerve-to-nerve suture group was somewhat smaller than might be expected when compared to the nerve graft and muscle graft groups. A comparison of the percentage recovery of mean peak twitch tension between non-diabetic and diabetic groups is useful because the differences in muscle tension which result from variations in animal size due to the diabetic state are compensated for. Similar degrees of recovery were obtained by the diabetic animals when the mean peak twitch tension of each operated group was expressed as a percentage of the mean peak twitch tension of the diabetic control group. The percentage recovery of peak twitch tension of the diabetic crush group was 78.51% and 49.57% for the diabetic nerve-to-nerve suture group. The

respective values for the diabetic nerve graft and muscle graft groups were 52.89% and 32.64%.

Results from significance tests on the values of maximum isometric twitch tension (indirect stimulation).

a) F test for variance.

The value obtained for F from performing the F test on the values of maximum isometric twitch tension was $F = 5.6$, d.f. = 9, 70. This was highly significant ($p < 0.001$) which indicated that the samples were not from the same population.

b) Student's t tests on the means of maximum isometric twitch tension.

Non-diabetic rats.

Table 6.3 shows the values of t and the significance levels of the Student's t test performed on the means of maximum isometric twitch tension that were obtained from the non-diabetic animals. The values of mean maximum isometric twitch tension of the operated groups were significantly smaller than the mean of the control group. The mean peak twitch tension of the nerve crush group was different from the mean of the control group at the 5% level, whereas, the mean peak twitch tensions of the nerve-to-nerve suture, nerve graft and muscle graft groups were significantly smaller than the control group mean at the 1% level. This indicates that recovery of maximum isometric twitch tension after nerve crush was more complete at 150 days than the recovery of the other operated groups after the same length of time. The recovery of mean peak twitch tension of the operated groups as compared with each other were not significantly different ($p > 0.05$).

Table 6.3 Table of t values and significance levels for the values of maximum isometric twitch tension of the soleus muscle (indirect stimulation) in non-diabetic animals (Student's t test)

	C	CR	NN	NG	MG
C	-	$t=3.29$ $p<0.05$	$t=5.72$ $p<0.01$	$t=4.41$ $p<0.01$	$t=4.35$ $p<0.01$
CR	-	-	$t=2.83$ NS	$t=1.71$ NS	$t=1.64$ NS
NN	-	-	-	$t=-0.63$ NS	$t=-0.70$ NS
NG	-	-	-	-	$t=-0.06$ NS
MG	-	-	-	-	-

Diabetic rats.

Table 6.4 shows the values of t and the significance levels of the Student's t test performed on the means of maximum isometric twitch tension that were obtained from the diabetic animals. In contrast to the findings for the non-diabetic animals, the mean peak twitch tension of each of the operated groups of animals was not significantly different from the diabetic control mean. In fact, no significant difference was found between the means of any combination of groups ($p>0.05$). These results demonstrate that the recovery of tension after nerve injury and repair in diabetic animals is good.

Table 6.4 Table of t values and significance levels for the values of maximum isometric twitch tension of the soleus muscle (indirect stimulation) in diabetic animals (Student's t test)

	DC	DCR	DNN	DNG	DMG
DC	-	t=0.91 NS	t=2.08 NS	t=1.88 NS	t=2.43 NS
DCR	-	-	t=1.23 NS	t=1.05 NS	t=1.69 NS
DNN	-	-	-	t=-0.13 NS	t=0.6 NS
DNG	-	-	-	-	t=0.7 NS
DMG	-	-	-	-	-

Diabetic *v* non-diabetic rats.

It can be seen from table 6.5 which shows the values of t and the significance levels for t tests performed on the means of maximum isometric twitch tension of like groups from the diabetic and non-diabetic populations, that the values of mean peak twitch tension did not differ significantly in diabetic from non-diabetic animals ($p > 0.05$). However, it should be noticed that the t test performed on the means of peak twitch tension of both control groups approached significance ($t = 3.57$). Had the t tests not been adjusted for making multiple comparisons, this would have been a significant result. The difference in the peak twitch tension produced by the soleus muscle in diabetic and non-diabetic animals could be explained by a difference in the mean size of the muscle from the two different populations.

Table 6.5 Table of t values and significance levels for the values of maximum isometric twitch tension of the soleus muscle (indirect stimulation), non-diabetic animals *v* diabetic animals (Student's t test)

	C	CR	NN	NG	MG
DC	t=3.57 NS	-	-	-	-
DCR	-	t=1.37 NS	-	-	-
DNN	-	-	t=-0.13 NS	-	-
DNG	-	-	-	t=0.38 NS	-
DMG	-	-	-	-	t=1.06 NS

6.3.4 Results from the Measurement of Maximum Isometric Tetanic Tension.

The values of isometric tetanic tension, mass of the left and right soleus muscles and the ratio of left/right soleus muscle mass are displayed in appendix 17. In each group of animals n = 5. The residuals of isometric tetanic tension were plotted against the fitted values of the mean of each group which showed that the variance of each group was similar. Normal and half-normal plots were constructed which demonstrated the data to be normally distributed with no outliers.

Tables 6.6 and 6.7 show the values of the mean, standard deviation, standard error of the mean and the coefficient of variation of isometric tetanic tension for each group of non-diabetic and diabetic animals, respectively. From these tables it can be seen that the tensions produced by the soleus muscle in the diabetic animals were smaller than the tensions produced by the non-diabetic animals. However, the values of mean tetanic

tension of each operated group expressed as a percentage of the mean tetanic tension of the control group were not dissimilar for non-diabetic and diabetic animals. Both the diabetic and non-diabetic crush groups recovered tetanic tensions comparatively better than the other operated groups after 150 days. In fact, the mean tetanic tension for the diabetic crush group was bigger than the mean tension of the diabetic control group, although the mean tetanic tension of the diabetic crush group was very similar in size to the mean tetanic tension of the non-diabetic crush group.

Table 6.6 Values of the mean, standard deviation, standard error of the mean and coefficient of variation of tetanic tension of the soleus muscle in non-diabetic animals

RAT GROUP	n	m /N	SD	SEM	c.v.
C	5	0.97	0.51	0.23	52.74
CR	5	0.78	0.43	0.19	54.45
NN	5	0.30	0.12	0.05	39.87
NG	5	0.28	0.11	0.05	38.87
MG	5	0.39	0.09	0.04	23.89

Table 6.7 Values of the mean, standard deviation, standard error of the mean and coefficient of variation of tetanic tension of the soleus muscle in diabetic animals

RAT GROUP	n	m /N	SD	SEM	c.v.
DC	5	0.57	0.21	0.10	37.47
DCR	5	0.72	0.37	0.17	51.08
DNN	5	0.23	0.10	0.04	43.56
DNG	5	0.22	0.15	0.07	68.67
DMG	5	0.22	0.06	0.03	29.31

Results from significance tests on the values of maximum isometric tetanic tension.

a) F test for variance.

The F test performed on the values of tetanic tension gave a significant value of F = 4.35, d.f = 9,49. This test showed that the samples were unlikely to be from the same population.

b) Student's t tests on the means of isometric tetanic tension.

Non-diabetic rats.

Table 6.8 shows the t values and the significance levels of the t tests performed on the mean values of tetanic tension produced by the soleus muscles of the different groups of non-diabetic animals. The mean tetanic tension for the nerve-to-nerve suture, nerve graft and muscle graft groups was significantly smaller than the mean tetanic tension of the control group ($p < 0.05$). The mean tetanic tension for the crush group was 80.41% of the mean tetanic tension of the control group but they were not significantly different at the 5% level.

Table 6.8 Table of t values and significance levels for the values of tetanic tension of the soleus muscle in non-diabetic animals (Student's t test)

	C	CR	NN	NG	MG
C	-	t=1.04 NS	t=3.63 p<0.05	t=3.74 p<0.05	t=3.13 p<0.05
CR	-	-	t=2.59 NS	t=2.70 NS	t=2.10 NS
NN	-	-	-	t=0.11 NS	t=-0.50 NS
NG	-	-	-	-	t=-0.61 NS
MG	-	-	-	-	-

There was no significant difference in the recovery of tetanic tension after the different types of nerve injury and repair ($p > 0.05$).

Diabetic rats.

As was seen in the results of the t tests on the values of maximum isometric twitch tension, there was no significant difference between the means of tetanic tension in any combination of groups ($p > 0.05$). However, the mean values of tetanic tension in the nerve-to-nerve suture, nerve graft and muscle graft groups were only 40.35%, 38.60% and 38.60% of the mean of the diabetic control group, respectively. The values of t and the significance levels of the t tests performed on the means of tetanic tension of the diabetic groups are shown in table 6.9.

Table 6.9 Table of t values and significance levels for the values of tetanic tension of the soleus muscle in diabetic animals (Student's t test)

	DC	DCR	DNN	DNG	DMG
DC	-	t=-0.83 NS	t=1.84 NS	t=1.86 NS	t=1.88 NS
DCR	-	-	t=2.66 NS	t=2.68 NS	t=2.7 NS
DNN	-	-	-	t=0.02 NS	t=0.04 NS
DNG	-	-	-	-	t=0.91 NS
DMG	-	-	-	-	-

Diabetic *v* non-diabetic rats.

As in the results of the t tests on the values of maximum isometric twitch tension, there was no significant difference between the means of tetanic tension of like groups of diabetic and non-diabetic animals ($p > 0.05$).

Table 6.10 shows the values of t and the significance levels for t tests performed on the means of maximum tetanic tension in like groups in the diabetic and non-diabetic populations. It can be seen from table 6.10 that the biggest difference between like groups of non-diabetic and diabetic animals was that between control and crush groups.

Table 6.10 Table of t values and significance levels for the values of tetanic tension of the soleus muscle, non-diabetic animals *v* diabetic animals (Student's t test)

	C	CR	NN	NG	MG
DC	t=2.17 NS	-	-	-	-
DCR	-	t=2.59 NS	-	-	-
DNN	-	-	t=0.38 NS	-	-
DNG	-	-	-	t=0.29 NS	-
DMG	-	-	-	-	t=0.91 NS

6.3.5 Results from the Measurement of Maximum Isometric Twitch Tension (direct stimulation).

Table 6.11 an 6.12 show the values of the mean, standard deviation, standard error of the mean and coefficient of variation of mean maximum isometric twitch tension (direct stimulation) from non-diabetic and diabetic rats, respectively. The variance of each group of values of maximum isometric twitch tension was found to be similar using plots of the residual values against their fitted values (see chapter 4.3.1). Normal and half-normal plots were constructed from the residual and absolute residual values,

which were plotted against the expected normal quantiles. The plots formed straight lines and, therefore, provided justification for using parametric statistical analysis.

Table 6.11 Values of the mean, standard deviation, standard error of the mean and coefficient of variation of maximum isometric twitch tension of the soleus muscle (direct stimulation) in non-diabetic animals

RAT GROUP	n	m /N	SD	SEM	c.v.
C	5	0.25	0.06	0.03	22.50
CR	7	0.14	0.06	0.02	38.94
NN	10	0.13	0.05	0.02	38.35
NG	5	0.17	0.07	0.03	43.44
MG	5	0.17	0.03	0.01	16.35

Table 6.12 Values of the mean, standard deviation, standard error of the mean and coefficient of variation of maximum isometric twitch tension of the soleus muscle (direct stimulation) in diabetic animals

RAT GROUP	n	m /N	SD	SEM	c.v.
DC	8	0.16	0.06	0.02	40.79
DCR	9	0.17	0.05	0.02	27.85
DNN	8	0.18	0.07	0.02	39.23
DNG	7	0.12	0.04	0.02	34.90
DMG	5	0.12	0.01	0.01	11.00

The mean values of maximum isometric twitch tension of each group, after direct stimulation, were closer to each other than after indirect stimulation (see tables 6.1 and 6.2, for non-diabetic and diabetic rats, respectively). There was no obvious pattern of size distribution of mean twitch tension (direct stimulation), however, the mean values obtained after nerve graft and muscle graft, in both non-diabetic and diabetic animals, were equal in magnitude and smaller than their respective controls. As in mean twitch tension of the non-diabetic control group (indirect stimulation),

the value after direct stimulation was much larger than any of the other means.

Results from significance tests on the values of maximum isometric twitch tension (direct stimulation).

a) F test for variance.

The value of F obtained from analysis of variance performed on the data accumulated of maximum isometric twitch tension (direct stimulation) gave a value of $F = 2.43$, d.f. = 9,70. This result was significant ($p < 0.02$).

b) Student's t tests on the means of maximum isometric twitch tension (direct stimulation).

Non-diabetic rats.

The values of t and the significance levels for the t tests performed on the means of maximum isometric twitch tension (direct stimulation), from non-diabetic animals, are displayed in table 6.13. Both the crush and nerve-to-nerve suture groups produced means of twitch tension that were significantly smaller than the control group mean ($p < 0.05$). The mean values of tension obtained from the nerve graft and muscle graft groups (0.17 N, for both groups) were smaller than the mean of the control group (0.25 N) but the values were not significantly different from each other ($p > 0.05$), although the values of t approached the level of significance. No other combination of means showed any significant difference ($p > 0.05$).

Diabetic rats.

The values of t and the significance levels for the t tests performed on the means of maximum isometric twitch tension (direct stimulation), of the diabetic animals, are diplayed in table 6.14. T tests on the group means

showed that there was no significant difference between any of the means of isometric twitch tension (direct stimulation) ($p > 0.05$).

Table 6.13 Table of t values and significance levels for the values of maximum isometric twitch tension of the soleus muscle (direct stimulation) in non-diabetic animals (Student's t test)

	C	CR	NN	NG	MG
C	-	t=3.28 p<0.05	t=3.81 p<0.05	t=2.412 NS	t=2.33 NS
CR	-	-	t=0.56 NS	t=-0.55 NS	t=-0.63 NS
NN	-	-	-	t=-1.03 NS	t=-1.11 NS
NG	-	-	-	-	t=-0.07 NS
MG	-	-	-	-	-

Table 6.14 Table of t values and significance levels for the values of maximum isometric twitch tension of the soleus muscle (direct stimulation) in diabetic animals (Student's t test)

	DC	DCR	DNN	DNG	DMG
DC	-	t=0.35 NS	t=-0.74 NS	t=1.09 NS	t=1.08 NS
DCR	-	-	t=-0.42 NS	t=1.45 NS	t=1.4 NS
DNN	-	-	-	t=1.81 NS	t=1.73 NS
DNG	-	-	-	-	t=0.08 NS
DMG	-	-	-	-	-

Non-diabetic *v* diabetic rats.

Table 6.15 shows the values of t and the significance levels from the t tests performed on the means of maximum isometric twitch tension (direct stimulation) from like groups of non-diabetic and diabetic animals.

No significant difference was found between any of the means of twitch tension when t tests were performed on pairs of means from like groups of non-diabetic and diabetic animals ($p > 0.05$). However, the value of t obtained from a t test on the means of twitch tension from non-diabetic control and diabetic control groups was $t = 2.99$. This value was very different from those obtained from the t tests on the means of twitch tension of paired crush, nerve-to-nerve suture, nerve graft and muscle graft groups, which were $t = -0.62, -1.58, 1.28, 1.33$, respectively. This result showed that the biggest difference in maximum isometric twitch tension (direct stimulation), when comparing non-diabetic and diabetic animals, was between control groups.

Table 6.15 Table of t values and significance levels for the values of maximum isometric twitch tension of the soleus muscle (direct stimulation), non-diabetic animals *v* diabetic animals (Student's t test)

	C	CR	NN	NG	MG
DC	t=2.99 NS	-	-	-	-
DCR	-	t=-0.62 NS	-	-	-
DNN	-	-	t=-1.58 NS	-	-
DNG	-	-	-	t=1.28 NS	-
DMG	-	-	-	-	t=1.33 NS

6.3.6 Results from Determination of Mass of the Soleus Muscle.

The soleus muscle of each leg, from the same animals as were used in the isometric tetanic tension experiments, was removed and weighed to determine its mass. The muscles were not removed from the animals used in the isometric twitch experiments. The values of mass of the soleus muscle from both legs of each rat are displayed in appendix 17; the ratio of masses of left/right soleus muscle are also displayed in appendix 17. Tables 6.16 and 6.17 show the values of the mean, standard deviation, standard error of the mean and the coefficient of variation of the masses of the left soleus muscles of the different groups, of non-diabetic and diabetic animals, respectively. If these values are considered alongside the values of mean isometric tetanic tension which are displayed in tables 6.6 and 6.7, for non-diabetic and diabetic animals, respectively, it can be seen that the smaller values of mean tetanic tension were obtained from the groups where the values of mean mass of the left soleus muscle were also smaller. This indicates that the tension produced by the soleus muscle is dependent on muscle size and also that the differences between the tension developed by the soleus muscle after nerve injury and repair are possibly due to atrophic changes and not to incomplete reinnervation.

Table 6.16 Values of the mean, standard deviation, standard error of the mean and coefficient of the mass of the left soleus muscle in non-diabetic animals

RAT GROUP	n	m /g	SD	SEM	c.v.
C	5	0.33	0.02	0.01	5.35
CR	5	0.29	0.05	0.02	16.41
NN	5	0.23	0.04	0.02	19.50
NG	5	0.26	0.05	0.02	19.21
MG	5	0.20	0.05	0.02	23.51

Table 6.17 Values of the mean, standard deviation, standard error of the mean and coefficient of variation of the mass of the left soleus muscle in diabetic animals

RAT GROUP	n	m /g	SD	SEM	c.v.
DC	5	0.30	0.04	0.02	14.90
DCR	5	0.28	0.04	0.02	12.72
DNN	5	0.24	0.03	0.01	11.48
DNG	5	0.19	0.01	0.00	4.30
DMG	5	0.15	0.03	0.01	21.08

The ratio of masses was considered a useful measurement to make when comparing diabetic and non-diabetic animals. This is because an expression of the mass of the left soleus muscle as a ratio of the right soleus muscle eliminates the problem of differences in mass of the muscle due to differences in size of the animals. This is, of course, very important when considering diabetic and non-diabetic animals where the size of the animals varies considerably (see chapter 3). The values of the mean, standard deviation, standard error of the mean and coefficient of variation of the ratios of masses of left/right soleus muscle are diplayed in table 6.18 and 6.19 for non-diabetic and diabetic groups, respectively. As expected, the ratio of masses of both the non-diabetic control group and diabetic control group approximated to unity which is evidence that both left and right soleus muscles were of similar mass in unoperated animals. The fact that the ratio of masses of the diabetic and non-diabetic crush groups both approximated to unity and, in fact, were slightly greater than 1, suggests that hypertrophy of the soleus muscles on the unoperated sides had not occurred. From table 6.18 it can be seen that the ratio of masses was smaller for the nerve-to-nerve suture, nerve graft and muscle graft groups than for the control and crush groups; this also holds true for the diabetic animals (table 6.19).

Table 6.18 Values of the mean, standard deviation, standard error of the mean and coefficient of variation of the ratio of masses of left/right soleus muscle in non-diabetic animals

RAT GROUP	n	m	SD	SEM	c.v.
C	5	0.94	0.12	0.05	12.39
CR	5	1.02	0.22	0.10	21.90
NN	5	0.73	0.15	0.07	19.83
NG	5	0.80	0.06	0.03	7.12
MG	5	0.70	0.17	0.08	24.68

Table 6.19 Values of the mean, standard deviation, standard error of the mean and coefficient of variation of the ratio of masses of left/right soleus muscle in diabetic animals

RAT GROUP	n	m	SD	SEM	c.v.
DC	5	1.10	0.10	0.05	9.20
DCR	5	1.22	0.18	0.08	14.83
DNN	5	0.84	0.07	0.03	8.72
DNG	5	0.72	0.05	0.02	6.94
DMG	5	0.58	0.09	0.04	14.86

The residual values of the mass of the left soleus muscle were plotted against the different group means which showed that the variance of each group was similar. Straight lines were obtained from normal and half-normal plots made from the residual values of the mass of the left muscles and their expected values should the distribution be normal; no outliers were found. It was then considered appropriate to use parametric statistical analysis.

Results from significance tests on the values of mass of the left soleus muscle.

a) F test for variance.

The value of F obtained from analysis of variance performed on the values of of mass of the left soleus muscle was $F = 12.32$, d.f. = 9,49. This result was highly significant ($p < 0.01$).

b) Student's t tests on the values of mass of the left soleus muscle.

Non-diabetic rats.

Table 6.20 shows the values of t and significance levels for the t tests performed on the means of mass of the left soleus muscle in the various groups of non-diabetic rats. It can be seen from table 6.20 that the mean mass of the left soleus muscle of the muscle graft group was significantly smaller than the mean of the control group ($p<0.01$) and also significantly smaller than the mean of the nerve crush group ($p<0.05$). Although the mean of the nerve graft group was smaller than the mean of the control group ($m = 0.258$ g as compared with $m = 0.326$ g, they were not significantly different at the 5% level. However, the mean mass of the left soleus muscle of the nerve-to-nerve suture group was significantly less than the mean mass of the control group ($p<0.05$). There were no other differences in the mean mass of the left soleus muscle that were significant ($p>0.05$), however, it can be seen from table 6.18 that the mean of the control group was the biggest ($m = 0.326$ g). Of the operated groups, the mean mass of the left soleus muscle was largest in the crush group ($m = 0.292$ g).

Table 6.20 Table of t values and significance levels for the values of mass of the left soleus muscle in non-diabetic animals (Student's t test)

	C	CR	NN	NG	MG
C	-	t=1.27 NS	t=3.65 p<0.05	t=2.54 NS	t=4.62 p<0.01
CR	-	-	t=2.39 NS	t=1.27 NS	t=3.36 p<0.05
NN	-	-	-	t=-1.12 NS	t=0.97 NS
NG	-	-	-	-	t=2.09 NS
MG	-	-	-	-	-

Diabetic rats.

Table 6.21 shows the values of t and significance levels for the t tests performed on the means of mass of the soleus muscle in the various groups of diabetic rats. From table 6.21, it can be seen that the mean mass of the left soleus muscle of the muscle graft group was significantly smaller then the mean mass of the diabetic crush and control groups ($p < 0.01$) and also significantly smaller than the mean mass of the diabetic nerve-to-nerve suture group ($p < 0.05$). Although the mean mass of the left soleus muscle in the muscle graft group was smaller than the mean of the nerve graft group ($m = 0.150$ g as compared with $m = 0.186$ g), these values were not significantly different at the 5% level. The value of mean mass of the left soleus muscle in the diabetic nerve graft group was significantly smaller than the mean of the diabetic nerve crush ($p < 0.05$) and diabetic control group ($p < 0.01$). Although the mean mass of the diabetic nerve-to-nerve suture group was greater than the mean of the nerve graft group ($m = 0.244$ g as compared with $m = 0.186$ g), the values were not significantly different at the 5% level. The mean mass of the left soleus muscle in the diabetic nerve-to-nerve suture group was not significantly smaller than the means of either the diabetic nerve crush or the diabetic control groups ($p > 0.05$).

The residual values of the ratio of left/right soleus muscle mass were plotted against the different group means which showed the variance of each group to be similar. Straight lines were obtained from normal and half-normal plots made from the residual values of left/right soleus mass and their expected values showed the distribution be normal; no outliers were found. It was then considered appropriate to use parametric statistical analysis.

Table 6.21 Table of t values and significance levels for the values of the mass of the soleus muscle in diabetic animals (Student's t test)

	DC	DCR	DNN	DNG	DMG
DC	-	t=0.45 NS	t=1.94 NS	t=4.10 p<0.01	t=5.44 p<0.01
DCR	-	-	t=1.49 NS	t=3.65 p<0.05	t=5.00 p<0.01
DNN	-	-	-	t=2.16 NS	t=3.51 p<0.05
DNG	-	-	-	-	t=1.34 NS
DMG	-	-	-	-	-

a) F test for variance.

The value of F obtained from analysis of variance performed on the values of the ratio of masses of left/right soleus muscle was F = 9.12, d.f. = 9,49. This result was significant ($p<0.01$). Student's t tests were performed on the means of the ratio of like groups of non-diabetic and diabetic animals to see if the significant difference between sample means detected by the test for variance was due to differences between like groups of non-diabetic and diabetic animals.

b) Student's t tests on the means of the ratio of masses of left/right soleus muscle.

Non-diabetic *v* diabetic rats.

No significant difference was found between the means of the ratio of masses of left/right soleus muscle obtained from like groups of non-diabetic and diabetic animals ($p>0.05$). This indicates that the proportionate decrease in muscle mass observed after nerve injury and repair was no greater in the diabetic animals than in the non-diabetic animals. Table 6.22

shows the values of t and the significance levels for the t tests performed on the means of the ratio of masses of left/right soleus muscle

Table 6.22 Table of t values and significance levels for the ratio of masses of left/right soleus muscle, non-diabetic animals *v* diabetic animals (Student's t test)

	C	CR	NN	NG	MG
DC	t=-1.63 NS	-	-	-	-
DCR	-	t=-2.19 NS	-	-	-
DNN	-	-	t=-1.09 NS	-	-
DNG	-	-	-	t=0.77 NS	-
DMG	-	-	-	-	t=1.35 NS

6.3.7 Results from the Data of the Time to Peak Tension, Time to Half Peak Tension, Time to Half Relaxation and Duration of Isometric Twitch (indirect stimulation).

Normal and half-normal plots were constructed as described in chapter 4.3.1 for the data on each of: the time to peak tension, the time to half peak tension, the time to half relaxation and the duration of isometric twitch tension. The plots to determine how the variance of each group varied with the mean were made for each of these variables as described in chapter 4.3.1. The data obtained for each variable were found to be normally distributed, however, outliers were found in some groups. Where outliers were found they were eliminated from the complete set of results as they did not fit a normal distribution. The construction of normal and half-normal plots was then repeated. Outliers of each variable are reported in the appropriate section.

Results from significance tests on the values of time to peak tension (indirect stimulation).

One outlier was found in the non-diabetic nerve graft group. The value recorded from this rat was 109 ms. Table 6.23 and 6.24 display the values of the mean, standard deviation, standard error of the mean and coefficient of variation for the values of time to peak tension for non-diabetic and diabetic animals, respectively. It can be seen from these tables that very similar times to peak tension were obtained from diabetic and non-diabetic animals and there was no obvious pattern of difference between different treated and control groups. A comparison of tables 6.23 and 6.24 shows that the time to peak twitch tension in the groups of diabetic animals was, in general, slightly longer than the time to peak tension of the equivalent group of non-diabetic animals.

Table 6.23 Values of the mean, standard deviation, standard error of the mean and coefficient of variation of the time to peak tension (indirect stimulation) in non-diabetic animals

RAT GROUP	n	m /s	SD	SEM	c.v.
C	5	0.050	0.005	0.002	9.45
CR	7	0.045	0.006	0.002	12.67
NN	10	0.042	0.006	0.002	14.46
NG	4	0.043	0.015	0.007	33.78
MG	5	0.050	0.006	0.003	12.40

Table 6.24 Values of the mean, standard deviation, standard error of the mean and coefficient of variation of the time to peak tension (indirect stimulation) in diabetic animals

RAT GROUP	n	m /s	SD	SEM	c.v.
DC	8	0.055	0.005	0.002	8.62
DCR	9	0.048	0.005	0.002	10.23
DNN	8	0.050	0.008	0.003	15.52
DNG	7	0.048	0.006	0.002	13.19
DMG	5	0.044	0.002	0.001	4.43

a) F test for variance.

The F test performed on the values of time to peak tension gave a value of F = 1.50, d.f. = 9,69. This was not significant at the 5% level and, consequently, the null hypothesis that there is no difference in the time to peak tension between different groups and between non-diabetic and diabetic animals was accepted.

Results from significance tests on the values of time to half peak tension (indirect stimulation).

The values of the mean, standard deviation, standard error of the mean and coefficient of variation of the time to half peak tension for non-diabetic and diabetic animals are displayed in tables 6.25 and 6.26, respectively. Similar to the results for the time to peak tension, the values of

time to half peak tension obtained from the diabetic animals were, in general, very slightly longer than the values obtained from the non-diabetic animals. The non-diabetic control group mean was m = 0.030 s and the diabetic control group mean was m = 0.032 s. Both the diabetic muscle graft group and the non-diabetic muscle graft group had equal but smaller means of time to half peak tension than the control means (m = 0.022 s in each case). There was only one operated group mean that was larger than the control mean and that was the diabetic nerve-to-nerve suture group which had a mean time to half peak tension of m = 0.033 s.

Table 6.25 Values of the mean, standard deviation, standard error of the mean and coefficient of variation of the time to half peak tension (indirect stimulation) in non-diabetic rats

RAT GROUP	n	m /s	SD	SEM	c.v.
C	5	0.030	0.003	0.002	11.62
CR	7	0.026	0.004	0.002	16.92
NN	10	0.026	0.005	0.002	19.99
NG	5	0.020	0.006	0.003	31.68
MG	5	0.022	0.005	0.002	24.41

Table 6.26 Values of the mean, standard deviation, standard error of the mean and coefficient of variation of the time to half peak tension (indirect stimulation) in diabetic rats

RAT GROUP	n	m /s	SD	SEM	c.v.
DC	8	0.032	0.005	0.002	15.25
DCR	9	0.029	0.003	0.001	11.38
DNN	8	0.033	0.010	0.004	30.36
DNG	7	0.026	0.002	0.001	8.78
DMG	5	0.022	0.019	0.008	59.68

a) F test for variance.

The value of F obtained for the F test performed on the data of time to half peak tension was F = 1.86, d.f. = 9,70. This was not significant at the 5% level.

Results from the data of the values of time to half relaxation (indirect stimulation).

The values of the mean, standard deviation, standard error of the mean and coefficient of variation of the time to half relaxation of the non-diabetic and diabetic animals are displayed in table 6.27 and 6.28, respectively. From the standard deviations and coefficients of variation (tables 6.27 and 6.28), it can be seen that there was a large range in the values of time to half relaxation within each group. Although the data appeared to be normally distributed, there was too much variation in the data and too many outliers to make parametric statistical analysis feasible. Because the values of duration of isometric twitch provide similar information about the twitch to the time to half relaxation, no further tests were performed on the data of time to half relaxation.

Table 6.27 Values of the mean, standard deviation, standard error of the mean and coefficient of variation of the time to half relaxation from the time to peak tension (indirect stimulation) in non-diabetic animals

RAT GROUP	n	m /s	SD	SEM	c.v.
C	5	0.022	0.002	0.001	7.21
CR	7	0.025	0.004	0.002	17.39
NN	10	0.019	0.007	0.002	36.82
NG	5	0.086	0.084	0.037	96.91
MG	5	0.041	0.012	0.005	29.21

Table 6.27 Values of the mean, standard deviation, standard error of the mean and coefficient of variation of the time to half relaxation from the time to peak tension (indirect stimulation) in diabetic animals

RAT GROUP	n	m /s	SD	SEM	c.v.
DC	8	0.0400	0.043	0.015	109.13
DCR	9	0.0567	0.063	0.021	111.86
DNN	7	0.0233	0.007	0.003	29.90
DNG	7	0.0487	0.029	0.011	58.69
DMG	5	0.0360	0.008	0.004	22.57

Results from the data of the values of duration of isometric twitch (indirect stimulation).

Tables 6.29 and 6.30 show the values of the mean, standard deviation, standard error of the mean and coefficient of variation of the values of duration of muscle twitch for non-diabetic and diabetic rats, respectively. It can be seen from these tables that the duration of the twitch contraction was very similar in both diabetic and non-diabetic animals. It is also apparent from these tables that the length of contraction was longest in both of the muscle graft groups (m = 0.41 s in both cases). The mean duration of twitch contraction of both the non-diabetic and diabetic nerve-to-nerve suture groups was shorter than their respective control groups, whereas all of the other operated groups had mean durations of twitch contraction that were longer than the control means.

a) F test for variance.

The value of F obtained from the test for variance on the values of duration of twitch contraction was F = 2.19, d.f. = 9,70. The significance level of the F test was p = 0.035. This value of p demonstrates that the groups of animals are unlikely to be from the same population.

Table 6.29 Values of the mean, standard deviation, standard error of the mean and coefficient of variation of the duration of muscle twitch (indirect stimulation) in non-diabetic animals

RAT GROUP	n	m /s	SD	SEM	c.v.
C	5	0.26	0.06	0.03	21.95
CR	7	0.27	0.07	0.03	25.55
NN	10	0.21	0.05	0.02	24.82
NG	5	0.38	0.29	0.13	76.32
MG	5	0.41	0.07	0.03	16.28

Table 6.30 Values of the mean, standard deviation, standard error of the mean and coefficient of variation of the duration of muscle twitch (indirect stimulation) in diabetic animals

RAT GROUP	n	m /s	SD	SEM	c.v.
DC	8	0.29	0.08	0.03	27.73
DCR	9	0.49	0.26	0.09	53.63
DNN	8	0.26	0.16	0.06	60.46
DNG	7	0.43	0.17	0.06	39.38
DMG	5	0.41	0.07	0.03	17.81

b) Student's t tests on the means of duration of isometric twitch.

Adjusted t tests are used when multiple t tests are performed on the same data. The level of t that produces a significant result is necessarily higher than in single t tests to allow for type I error. Consequently, the t tests performed on the duration of isometric twitch using Scheffé's method did not show any significant difference between any combination of group means of duration of isometric twitch ($p > 0.05$).

6.3.8 Results from the Measurement of Time-tension Integral (indirect stimulation).

The values of the mean, standard deviation, standard error of the mean and coefficient of variation of the time-tension integral of non-diabetic and diabetic animals are displayed in table 6.31 and 6.32, respectively. The values of mean time-tension integral of the non-diabetic control and crush groups were much larger than the values of mean time-tension integral of the nerve-to-nerve suture, nerve graft and muscle graft groups. The values of the mean time-tension integral of the diabetic control and diabetic crush groups were also larger than the values of mean time-tension integral of the other three diabetic groups, however, the difference between groups was less for the diabetic than the non-diabetic animals. Considering the fact that the mean duration of the isometric twitch did not differ between non-diabetic and diabetic groups of animals (tables 6.29 and 6.30 for non-diabetic and diabetic animals, respectively), it is most likely that any difference in mean time-tension integral between non-diabetic and diabetic groups was due to a difference in the force produced by the soleus muscles of the rats in these two populations.

Table 6.31 Values of the mean, standard deviation, standard error of the mean and coefficient of variation of the time-tension integral of the soleus muscle (indirect stimulation) in non-diabetic animals

RAT GROUP	n	m /N s	SD	SEM	c.v.
C	5	0.21	0.05	0.02	23.84
CR	7	0.17	0.06	0.02	36.93
NN	10	0.04	0.02	0.01	49.07
NG	5	0.07	0.04	0.02	65.73
MG	5	0.07	0.02	0.01	28.33

Table 6.32 Values of the mean, standard deviation, standard error of the mean and coefficient of variation of the time-tension integral of the soleus muscle (indirect stimulation) in diabetic animals

RAT GROUP	n	m /N s	SD	SEM	c.v.
DC	8	0.14	0.03	0.01	20.89
DCR	9	0.17	0.07	0.02	41.98
DNN	8	0.08	0.05	0.02	65.94
DNG	7	0.10	0.02	0.01	21.56
DMG	5	0.08	0.01	0.01	18.66

Normal and half-normal plots were constructed from the residuals of the time-tension integral plotted against their expected values should they be normally distributed. The data was found to be normally distributed and the variance of each group was found to be similar. This provided justification for performing parametric statistical analysis on the data of time-tension integral.

Results from statistical tests on the values of time-tension integral (indirect stimulation).

a) F test for variance.

The value of F obtained from analysis of variance on the data of time-tension integral was $F = 9.02$, d.f. = 9,70. This result was highly significant ($p<0.001$) demonstrating that the groups of animals were unlikely to be from the same population.

b) Student's t tests on the means of time-tension integral.

Non-diabetic rats.

The values of t and the significance values from the t tests performed on the means of time-tension integral of the non-diabetic animals are displayed in table 6.33. The mean time-tension integral of the crush group was smaller than that of the control group (m = 0.17 and 0.21 N s), however, they were not significantly different ($p>0.05$). The means of the

nerve-to-nerve suture, nerve graft and muscle graft groups (m = 0.04, 0.07 and 0.07 N s, respectively) were all smaller than the mean time-tension integral of the control and crush groups. In fact, the mean time-tension integral of the nerve-to-nerve suture, nerve graft and muscle graft groups were significantly smaller than the means of the control and crush groups in every case ($p<0.01$). However, the mean time-tension integral of the nerve-to-nerve suture, nerve graft and muscle graft groups were not significantly different from each other ($p>0.05$).

Table 6.33 Table of t values and significance levels for the values of the time-tension integral (indirect stimulation) in non-diabetic animals (Student's t test)

	C	CR	NN	NG	MG
C	-	t=2.02 NS	t=6.52 p<0.01	t=4.79 p<0.01	t=4.75 p<0.01
CR	-	-	t=5.32 p<0.01	t=3.42 p<0.01	t=3.37 p<0.01
NN	-	-	-	t=-0.98 NS	t=-1.03 NS
NG	-	-	-	-	t=-0.04 NS
MG	-	-	-	-	-

Diabetic rats.

The values of t and the significance values from the t tests performed on the means of time-tension integral of the diabetic animals are displayed in table 6.34. Although the mean time-tension integral for each of the diabetic nerve-to-nerve suture, nerve graft and muscle graft groups (m = 0.08, 0.10 and 0.08 N s, respectively) was smaller than the mean of the control group (m = 0.17 N s), they were not significantly different at the 5% level. However, the mean time-tension integral of the diabetic muscle

graft group was sigificantly smaller than that of the diabetic crush group mean ($p<0.05$), as was the mean time-tension integral of the diabetic nerve-to-nerve suture group ($p<0.05$). None of the other means of time-tension integral was significantly different from any other ($p>0.05$).

Table 6.34 Table of t values and significance levels for the values of the time-tension integral (indirect stimulation) in diabetic animals (Student's t test)

	DC	DCR	DNN	DNG	DMG
DC	-	t=-1.11 NS	t=2.49 NS	t=1.67 NS	t=2.21 NS
DCR	-	-	t=3.67 p<0.05	t=2.79 NS	t=3.22 p<0.05
DNN	-	-	-	t=-0.73 NS	t=0.02 NS
DNG	-	-	-	-	t=0.67 NS
DMG	-	-	-	-	-

Non-diabetic *v* diabetic rats.

Table 6.35 shows the values of t and the levels of significance for the t tests performed on the means of time-tension integral measurements from like groups of non-diabetic and diabetic animals. There was no significant difference in the means of time-tension integral between non-diabetic and diabetic animals for any experimental group. However, the value of t obtained from the t test between the control and diabetic control groups was t = 2.63. This value was very different from the values of t obtained for the nerve crush, nerve-to-nerve suture, nerve graft and muscle graft groups (t = -0.36, -1.75, -1.14 and -0.40, respectively. It has been argued (see above) that the greater difference in mean time-tension integral seen

between non-diabetic and diabetic control groups, as compared to the other non-diabetic and diabetic groups, was due to the greater difference in tension produced by the two control groups. The results of section 6.3.6 showed that the difference in muscle size between diabetic and non-diabetic animals was greatest in the control and crush groups. It is probable, therefore, that the greater differences between non-diabetic and diabetic animals in producing tension, and hence, greater differences in values of time-tension integral, were due to differences in muscle size. If after operating on the sciatic nerve, the difference in mass of the soleus muscle between diabetic and non-diabetic muscles was smaller (see section 6.3.6), then it is to be expected that differences in tension and in the time-tension integral would also be smaller when comparing like groups of non-diabetic and diabetic animals, if the degree of reinnervation was unaffected by the diabetic state.

Table 6.35 Table of t values and significance levels for the values of the time-tension integral (indirect stimulation), non-diabetic animals *v* diabetic animals (Student's t test)

	C	CR	NN	NG	MG
DC	t=2.63 NS	-	-	-	-
DCR	-	t=-0.36 NS	-	-	-
DNN	-	-	t=-1.75 NS	-	-
DNG	-	-	-	t=-1.14 NS	-
DMG	-	-	-	-	t=-0.4 NS

6.3.9 Results from the Data of the Time to Peak Tension and Duration of Isometric Twitch (direct stimulation).

The difficulty in stimulating muscle directly such that all of the fibres are activated, is discussed in chapter 6.4.3. Similar methods to those used in the work described in this thesis were used to stimulate rabbit muscle by Hems (1993) and to stimulate rat muscle by Myles (1990). Neither of these authors has reported the results of their direct stimulation experiments. The results of the direct stimulation experiments reported in this thesis will be discussed even though the intentions of the experiments have not been realized. The findings of the experiments to determine maximal isometric twitch tension produced by direct stimulation of the soleus muscle are presented in section 6.3.5. The results of the time course of the isometric twitch were not considered to be of particular interest and so will be discussed only briefly.

Results on the data of time to peak tension (direct stimulation).

Tables 6.36 and 6.37 show the values of the mean, standard deviation, standard error of the mean and the coefficient of variation of the time to peak tension for non-diabetic and diabetic animals, respectively. The mean time to peak tension of the different experimental groups was similar for both diabetic and non-diabetic animals. This finding was similar to that observed for the time to peak tension produced by indirect stimulation. The values of mean time to peak tension were similar for crush and control groups from both diabetic and non-diabetic populations. The mean time to peak tension of the nerve-to-nerve suture, nerve graft and muscle graft groups tended to be smaller than the control and crush group means, for non-diabetic and diabetic animals. The only exception was the mean time to peak tension of

the non-diabetic control group which was longer than the mean time to peak tension of the non-diabetic control group (m = 0.080 s as compared to m = 0.078 s).

Table 6.36 Values of the mean, standard deviation, standard error of the mean and coefficient of variation of the time to peak tension of the soleus muscle (direct stimulation) in non-diabetic animals

RAT GROUP	n	m /s	SD	SEM	c.v.
C	5	0.078	0.012	0.005	15.48
CR	7	0.078	0.017	0.006	21.17
NN	10	0.060	0.010	0.003	17.20
NG	5	0.080	0.028	0.012	34.46
MG	5	0.055	0.002	0.001	4.53

Table 6.37 Values of the mean, standard deviation, standard error of the mean and coefficient of variation of the time to peak tension of the soleus muscle (direct stimulation) in diabetic animals

RAT GROUP	n	m /s	SD	SEM	c.v.
DC	8	0.096	0.016	0.005	16.18
DCR	8	0.104	0.024	0.008	22.85
DNN	8	0.062	0.013	0.005	21.71
DNG	7	0.061	0.007	0.003	12.84
DMG	5	0.055	0.010	0.004	18.17

Results on the data of duration of the isometric twitch contraction (direct stimulation).

Tables 6.38 and 6.39 show the values of the mean, standard deviation, standard error of the mean and the coefficient of variation of the duration of the isometric twitch for non-diabetic and diabetic animals, respectively. The mean duration of the twitch of the crush and control groups was similar in both non-diabetic and diabetic animals. In general, the duration of the twitch tended to be shorter in the operated groups where nerve injury of the

neurotmesis type had been induced. The only exception was the mean duration of isometric twitch of the non-diabetic muscle graft group (m = 0.66 s) which was longer than the mean of non-diabetic control group (m = 0.60 s). Surprisingly, the values of mean duration of isometric twitch of the diabetic control and crush groups (m = 0.82 s and m = 0.83 s, respectively) were longer than the mean duration of the isometric twitch of the non-diabetic control and crush groups (m = 0.60 s and m = 0.61 s, respectively).

Table 6.38 Values of the mean, standard deviation, standard error of the mean and coefficient of variation of the duration of muscle twitch (direct stimulation) in non-diabetic animals

RAT GROUP	n	m /s	SD	SEM	c.v.
C	5	0.60	0.10	0.05	16.93
CR	7	0.61	0.20	0.08	33.16
NN	10	0.56	0.14	0.05	25.58
NG	5	0.57	0.25	0.11	43.33
MG	5	0.66	0.01	0.01	2.10

Table 6.39 Values of the mean, standard deviation, standard error of the mean and coefficient of variation of the duration of muscle twitch (direct stimulation) in diabetic animals

RAT GROUP	n	m /s	SD	SEM	c.v.
DC	8	0.82	0.06	0.02	7.06
DCR	9	0.83	0.12	0.04	14.03
DNN	8	0.51	0.11	0.04	22.24
DNG	7	0.48	0.15	0.06	31.77
DMG	5	0.58	0.13	0.06	22.85

6.3.10 Results from the Measurement of Time-tension Index (indirect stimulation).

The values of the mean, standard deviation, standard error of the mean and coefficient of variation of the time-tension index of the non-diabetic and diabetic animals are displayed in table 6.40 and 6.41 respectively. It can be seen from these tables that the mean time-tension index is largest for the control group of the non-diabetic animals (m = 0.87 N). The non-diabetic crush group produced a mean time-tension index of m = 0.66 N. The remaining three non-diabetic groups (nerve-to-nerve suture, nerve graft and muscle graft groups) produced much smaller but similar means for time-tension index (m = 0.19, 0.19 and 0.17 N, respectively). A similar pattern was seen in the diabetic animals where the means of the control and crush groups (m = 0.50 N and m = 0.46 N, respectively) were smaller than the values of the non-diabetic control animals but similar to each other and much larger than the mean time-tension index of the diabetic nerve-to-nerve suture, nerve graft and muscle graft groups (m = 0.22, 0.28 and 0.20 N, respectively).

Table 6.40 Values of the mean, standard deviation, standard error of the mean and coefficient of variation of the time-tension index of the soleus muscle (indirect stimulation) in non-diabetic animals

RAT GROUP	n	m /N	SD	SEM	c.v.
C	5	0.87	0.29	0.13	33.84
CR	7	0.66	0.24	0.09	37
NN	10	0.19	0.07	0.02	35.34
NG	5	0.19	0.08	0.04	43.47
MG	5	0.17	0.04	0.02	25.33

Table 6.41 Values of the mean, standard deviation, standard error of the mean and coefficient of variation of the time-tension index of the soleus muscle (indirect stimulation) in diabetic animals

RAT GROUP	n	m /N	SD	SEM	c.v.
DC	8	0.50	0.10	0.04	20.41
DCR	9	0.46	0.32	0.10	68.53
DNN	7	0.22	0.09	0.03	40.83
DNG	7	0.28	0.15	0.05	52.98
DMG	5	0.20	0.04	0.02	21.58

6.4 Discussion.

6.4.1 Recovery of Peak Isometric Twitch and Tetanic Tension.

The twitch/ tetanic ratio.

It can be seen from tables 6.1 and 6.2 (which show the mean, standard deviation, standard error of the mean and coefficient of variation of peak twitch tension in non-diabetic and diabetic rats, respectively) and tables 6.6 and 6.7 (which show the equivalent for tetanic tension in non-diabetic and diabetic rats, respectively) that the mean values of peak twitch tension were very much smaller than the mean values of tetanic tension for similar groups of animals. Although the experiments to find the maximum isometric twitch tension were performed on different animals from those that were used to find the isometric tetanic tension, a rough estimate of twitch/tetanic ratio can be made by dividing the mean maximum isometric twitch tension of the non-diabetic control group by the maximum isometric tetanic tension of the corresponding control group. The value obtained for the non-diabetic control animals was 0.50 and the equivalent value for the diabetic control animals was 0.42. Close (1965) found the ratio of maximum isometric twitch tension/tetanic tension of whole, rat extensor digitorum longus and soleus muscle, measured in vitro, to decrease during development. The ratios varied from 0.64, immediately after birth, to 0.19, 100 days after birth; measurements were made at 35-36 ^{o}C. In 1967, Close found the ratio of twitch tension/tetanic tension obtained from motor units of rat extensor digitorum longus and soleus muscles to range from 0.2 to 0.227 at 35 ^{o}C. In the work presented here, the experiments to measure

isometric tetanic tension were performed on different animals from those used in the experiments to measure isometric twitch tension and, consequently, differences in the amount of contractile tissue of the muscles in the two control groups may have been the cause of the large values for twitch/tetanic ratio. However, the estimates of twitch/tetanic ratio were similar for both the non-diabetic and diabetic animals. Notably, Close (1967) found that the peak twitch tension was almost doubled in the fast muscles of the rat at lower temperatures but the twitch tension of the slow soleus muscle was changed only slightly. This finding may be because the duration of the 'active state' is increased at lower temperatures, which allows greater time for stretching of the series elastic component and potentiation of the muscle twitch. The contraction time of the slower soleus muscle is already longer than that of the fast extensor digitorum longus (see below) and a decrease in temperature probably has less of an effect on the duration of the 'active state' in slow muscle and, therefore, the degree of potentiation of a twitch contraction of a slow muscle would also be less. Close (1967) also found that the isometric tetanic tension of rat muscles decreased with a decrease in temperature. Close (1972) stated in his review that the maximum tetanic tension produced in rat muscles, stimulated either directly or indirectly, at 35-37 ^{o}C was decreased by about 30% when the muscle was stimulated at 20 ^{o}C. Consequently, there is an inverse relation between the twitch/tetanus ratio and temperature, in the range of 20-37 ^{o}C. In the work presented in this thesis, the core temperature of the animals was kept as constant as possible by keeping the animals warm on an electrical blanket. However, it was noticed that if the the exposed soleus was touched, the temperature of the muscle dropped over the course of the experiments. Attempts were made to diminish the heat lost from the muscle, and any fall in the muscle temperature during the experiments, by wrapping the muscle

in cellophane and moistening the muscle with saline. The former probably had only a minimal effect in preventing heat loss from the muscle because great care had to be taken not to disrupt the experimental setup and, consequently, the muscle could not be insulated totally from the environment. The latter method of moistening the muscle with warm saline was found to exacerbate the problem of cooling of the muscle. This is because the evaporation of saline from the surface of the muscle caused even more heat loss. Since the temperature of the muscle during the experiments was well below 35-37 ^{o}C, and in view of the existence of the inverse relationship between twitch/tetanic ratio with temperature (see above), it is not surprising that the estimated values of the twitch/tetanic ratio were larger in the experiments presented here than in those of Close (1967) whose rat soleus muscles were more successfully kept at temperatures of between 35 and 37 ^{o}C. The methods of Close were very much more controlled than those of the work presented here; he was able to maintain constant temperature of the muscles by placing the dissected animal limb in a perspex bath containing Ringer solution at 35-37 ^{o}C, through which he continuously bubbled a gaseous mixture of 95% oxygen and 5% carbon dioxide.

Another factor that affects the development of maximal tetanic and twitch tension is the experimental problem of ensuring that the initial resting length of the muscle is optimum. Close (1972) stated that for twitch contractions, the optimum length was greater than that for tetanic contractions by 5-10%. In the work presented here, the same resting length was used in the experiments for recording both tetanic and twitch tensions (see chapter 6.2.3). The optimum length for recording isometric tetanic tension has been found to be 100-120% of the initial resting length (Close,1972). In the light of this evidence, it might be expected that the

isometric twitch tensions recorded in the experiments presented in this thesis would be slightly less than maximal. Consequently, it is thought that the rather large estimates of twitch/tetanic ratio of the experiments of this thesis may be, in part, due to different amounts of contractile tissue in the muscles of the twitch and tetanic tension experiments, but it is more likely that thay are due to a decrease in the tetanic tensions produced by the muscle and a slight potentiation of the twitch tensions produced in the experiments described in this thesis, as a result of the low temperature of the muscles. The individual values of twitch tension and tetanic tension are discussed below.

Isometric tetanic tension.

The difficulty in producing a standard for the measurement of force production in muscles, such that the force produced by one muscle can be compared directly with that of another, has been explained in the introduction. In the experiments presented here, measurements were made of the total tension developed in each soleus muscle as a result of stimulating the sciatic nerve with either a single supramaximal stimulus or a chain of stimuli. The size of the muscle is obviously of great importance when analysing the results (see chapter 6.1.5) particularly when comparing the tension produced by the soleus muscle of the smaller diabetic animals with the tension produced by the same muscle of the larger non-diabetic animals (chapter 3.3.1). It was for this reason that, after the electrophysiology had been performed, the soleus muscle was weighed to determine its mass.

When making comparisons between the measured values of tetanic tension, species difference, age difference, muscle type and size of muscle must all be taken into consideration. Close (1972) suggested that the huge

variation in the reported values of tetanic tension was a result of differences in methods of preparation. For example Sexton & Gersten (1967) measured the ATP-induced contraction of glycerol extracted fibres. Close (1967) found that the mean tetanic tension produced in the soleus muscle of six adult rats was 145±21 gwt; expressed in newtons this tension is approximately 1.42 N (taking acceleration due to gravity to be 9.8 m s^{-2}). The mean tetanic tension of the non-diabetic control group of the work presented here was only 0.97 N, however, the weights of the soleus muscles in the two experiments may have been very different.

Force produced by a muscle is directly proportional to its cross-sectional area and many authors express the tension produced by a muscle as tension per unit cross-sectional area. Close (1969) demonstrated the value of the tetanic tension per unit cross-sectional area of the female rat soleus muscle to be 1.9-2.1 kg cm^{-2}. If the average value of tension per unit cross-sectional area, as determined by the work of the aforementioned author, is taken to be 2.0 kg cm^{-2}, and is converted to N cm^{-2}, the value of the tetanic tension per unit cross-sectional area of the rat soleus muscle observed by Close (1969) was 19.6 N cm^{-2}. The mean tetanic tension obtained from the non-diabetic control animals of the work presented here was approximately 1 N. In order to obtain similar values of tension per unit cross-sectional area to those of Close (1969), the mean cross-sectional area of soleus muscle of the animals used in the experiments presented here would have had to have been 1/19.6 = 0.05 cm^2. An estimate of the mean cross-sectional area of the soleus muscle of rats used in the experiments presented here can be calculated: Mendez and Keys (1960) found the density of dog and rabbit muscle to be 1.06 g cm^{-3}. Using this value and the value of the mean soleus mass of the non-diabetic control group (0.33 g; see table 6.16), an estimate of the mean volume of the soleus

muscle used in the experiments of this thesis can be made using the equation below.

$$\text{volume} = \text{mass}/\ \text{density}$$

$$\text{mean volume} = 0.33/\ 1.06 = 0.311\ \text{cm}^3$$

To find the mean cross-sectional area, the following equation can be used:

$$\text{mean cross-sectional area} = \text{volume}/\ \text{length}$$

The soleus muscle was removed from the animal and laid on a piece of card. The length of the muscle was 5.5 cm. Inserting the values of mean volume and soleus muscle length into the equation above:

$$\text{mean cross-sectional area} = 0.311/5.5 = 0.057\ \text{cm}^2$$

The estimated mean cross-sectional area is very similar to that calculated above. From this reasoning, it is likely that the values of tetanic tension that were determined from the experiments in this thesis were similar to, if not, slightly smaller than those determined by Close (1969). Smaller values of tetanic tension, as compared to the tensions recorded by Close (1969), are most likely to have resulted from temperature differences in the muscles used in the experiments in this thesis and the muscles used in the experiments of Close (see above).

The results of the isometric tetanic tension experiments showed that the recovery of tetanic tension of the crush groups, from both non-diabetic

and diabetic populations, was better than the recovery after nerve transection and repair. This finding agrees with that of Berry *et al.* (1944) who showed almost complete restoration of the use of the operated limbs of cats after nerve crush injuries but after nerve-to-nerve suture, cats did not regain the normal use of the operated limb. The recovery of mean tetanic tension of the muscle and nerve graft groups was not dissimilar to the recovery of mean tetanic tension of the nerve-to-nerve suture groups, in both non-diabetic and diabetic animals. The values of mean tetanic tension in the crush groups did not differ significantly from, the control groups ($p > 0.05$ for both diabetic and non-diabetic animals), therefore indicating almost complete recovery after axonotmesis by 150 days. Recovery of tetanic tension after nerve grafting and muscle grafting were almost identical in the diabetic rats, and the recovery of tetanic tension in the non-diabetic animals was actually better after muscle grafting than after nerve grafting. These results seem to indicate that the recovery of tetanic tension is similarly poor after all nerve transection injuries regardless of the method of repair. The reason for the poorer recovery after nerve injury of the neurotmesis type, could be that there are fewer neuro-muscular connections being made after reinnervation which results from 'cross-wiring' of regenerating axons. However, Luff *et al.* (1988) demonstrated that full recovery of muscle force, after nerve injury in cats, could be produced with only 5% of motor axons intact. An alternative reason for the poorer recovery of tetanic tension after nerve transection injury could be because there is greater atrophy of muscle after neurotmesis. In the work described in this thesis, the mean mass of the soleus muscle for each of the operated groups of animals was smaller than that for the control group, and this was true for both non-diabetic and diabetic animals. The mean mass of the soleus muscle removed from the non-diabetic muscle

graft group was 61.96% of the mean mass of the soleus obtained from the non-diabetic control group. The mean tetanic tension of the non-diabetic muscle graft group was only 40.21% of the mean tetanic tension of the non-diabetic control group. The equivalent percentages for the diabetic muscle graft group were 50.67% of the mean mass of the soleus muscle of the diabetic control group and only 38.60% of the mean tetanic tension of the diabetic control group. These figures demonstrate that the poor recovery of tension after nerve injury is unlikely to be simply a matter of muscle atrophy. However, the difference in the mean tetanic tension produced by like groups of diabetic and non-diabetic animals may well have been due to differences in muscle size. The results of the non-diabetic and diabetic crush groups were very similar in terms of recovery of mean tetanic tension (m = 0.78 N and 0.72 N, for non-diabetic and diabetic rats, respectively). The mean mass of the soleus muscle for the two crush groups were also very similar (m = 0.29 g and 0.28 g for non-diabetic and diabetic rats ,respectively). In those groups where the mean tetanic tension of the diabetic animals was much lower than the mean for the non-diabetic animals, the mean mass of the soleus muscle was also much lower (see chapters 6.3.4 and 6.3.6). Statistical tests showed no significant difference in the recovery of tetanic tension between non-diabetic and diabetic animals ($p > 0.05$). Therefore, it appears that the recovery of muscle function after different types of nerve injury and repair is unaffected by the diabetic state and that any difference in the tetanic tension produced by non-diabetic and diabetic rat soleus muscle is due to muscle size.

Isometric twitch tension.

In some ways the value of twitch tension is not as useful an index of recovery of muscle function as the value of tetanic tension. This is because

the maximum tension that can be produced by a muscle is not produced in a twitch contraction (see chapter 6.1.4) and consequently, the values of twitch tension are much more variable with changes in the stimulus and the environment. The mean maximum isometric twitch tension of the extensor digitorum longus muscle of the adult rats used by Myles (1990) was approximately 0.59 N and the mean mass of the muscle was 0.24 g. The mean maximum isometric twitch tension of the non-diabetic control group of the work presented here was 0.481 N. Lewis and Ridge (1982) stated that the tension produced per unit cross-sectional area of fast-twitch muscles was twice that of slow-twitch muscles, therefore, it is to be expected that the twitch tension of extensor digitorum longus should be bigger than the twitch tension of the soleus muscle. Close (1972) quoted the maximum isometric twitch tension per unit cross-sectional area of the soleus muscle in adult rats to be 0.53 kg cm^{-2} and, in contrast to Lewis and Ridge (1982), he quoted the maximum isometric twitch tension per unit cross-sectional area of the extensor digitorum longus to be similar to that of soleus: 0.54 kg cm^{-2}. The mean maximum isometric tension of the non-diabetic control group in the work presented here was 0.48 N. If the same calculations as those used above are used to determine the mean cross-sectional area of the soleus muscle required to produced a twitch tension of 0.53 kg cm^{-2} the calculated mean cross-sectional area of the soleus muscle which produces a twitch tension of 0.48 N is 0.096 cm^2. This value of mean cross-sectional area is on the large side.

The twitch/tetanic ratio of the extensor digitorum longus muscle in adult rats was reported to be 0.19 by Close (1972) as compared with a ratio of 0.25 obtained from the soleus muscle at 35°C. The twitch/tetanic ratio and tetanic tension of the soleus muscles used in the work presented here have been discussed above. It was concluded that the tetanic tensions

recorded were perhaps a little smaller than might be expected but probably not small enough to cause such a large twitch/tetanic ratio as that estimated for the non-diabetic control animals. Therefore, it is most likely that the twitch tensions recorded in the experiments of this thesis were larger than might be expected. It is also likely that the values of twitch tension of rat extensor digitorum longus that were determined by Myles (1990) were also too large. Considering that the experiments of Myles (1990) and the experiments presented in this thesis were performed using the same equipment, it is likely that the same factors affected both sets of experiments. Potentiation of twitch tensions has been discussed in chapter 6.1.6. Staircase potentiation is unlikely to have occurred considering that Desmedt and Hainaut (1968) found twitch tension to have decreased after the first 10-20 twitches and, in the experiments in this thesis, the twitch tension was averaged over only 8 twitches. The experiments to find the tetanic tension were performed on different rats from those used in the twitch tension experiments and, therefore, post-tetanic potentiation was not a possible cause for the high values of twitch tension. The most likely explanation for the high values of twitch tension recorded in the experiments in this thesis is the low temperature of the muscle.

The difference in the values of mean maximum twitch tension between like groups of rats was similar to the difference in the values of mean tetanic tension between like groups of animals. The greatest difference between like groups of non-diabetic and diabetic animals was seen in the two control groups. The mean maximum twitch tension of the diabetic control group was 0.24 N and the value of t obtained from the Student's t test on the means of maximum twitch tension of the two control groups was $t = 3.57$. Using adjusted t levels for multiple t tests, this value of t was not significant at the 5% level, however, it was very different from the values of t obtained

from testing the means of like groups of the other non-diabetic and diabetic groups of animals (see table 6.5). The reason for this could be because the mean value of maximum twitch tension of the non-diabetic control group was abnormally large (see above). Alternatively, the difference in maximum twitch tension between the non-diabetic and diabetic control groups of animals could be because of a difference in the size of the muscles. Wasting of the soleus muscle after nerve injury and repair may have diminished the difference in twitch tension between the non-diabetic and diabetic animals that was due to size of the muscle and hence, the biggest difference seen between the non-diabetic animals and diabetic animals was in the control and crush groups.

6.4.2 Recovery of the Time Course of Twitch Contractions.

Time to peak tension.

The intrinsic speed of shortening of rat extensor digitorum longus is two and a half to three times faster than rat soleus muscle (Close, 1965; 1972). Myles (1990) found the mean time to peak tension of rat extensor digitorum longus was 21.5 ms and the mean time to half peak tension was 12.5 ms. The equivalent mean values for rat soleus muscle, determined from the experiments in this thesis were, 50 ms and 30 ms. The longer times to peak and to half peak tension of the soleus muscle, as compared with the extensor digitorum longus, are consistent with the differences in the type of muscle being investigated (see chapter 6.1.8). The absolute values of time to peak tension measured by Close (1965; 1967; 1972) are not comparable to those of the work in this thesis because he measured the time from the onset of the twitch to the peak of the twitch, whereas the values presented here, were measured from the time of stimulation to the time when the isometric twitch reached maximum tension. Close (1972) quoted the twitch contraction time of adult rat soleus muscle to be 36 ms and that of adult rat extensor digitorum longus to be 12.5 ms. The values of time to peak tension of the experiments presented here, and those of Myles (1990), were considerably slower than those of Close (1972) and cannot be explained totally by the additional time from initiation of stimulus to the onset of contraction. It should be noted that the control group of Myles (1990) consisted of animals varying in age by up to 300 days and, according to Close (1972), the speed of shortening of rat extensor digitorum longus decreases quite dramatically with age. Similar decreases in the speed of contraction of the rat soleus muscle do not occur and, consequently, any differences in the rate of contraction of rat soleus muscle between the

results of the experiments presented here and the work of other authors are unlikely to be due to maturational changes in the muscle. Close (1965) found that the time to peak tension (contraction time) of neonatal rat extensor digitorum longus approximately doubled for a decrease in temperature of 10 ^{o}C (from 35 ^{o}C to 25 ^{o}C). It is very likely that the longer contraction times recorded in the experiments in this thesis and by Myles (1990) are due to lower temperature of the muscle at the time of recording (see above).

The factors operating in the cross-innervation experiments of Buller *et al.* (1960a; 1960b) may be very relevant to the experiments described in this thesis where the whole sciatic nerve was transected and repaired (see chapter 6.1.13). This is because the motor fibres that initially innervated fast muscles (e.g. extensor digitorum longus) could have reinnervated the slow soleus muscle and caused changes in the fibre type of that muscle. Finkelstein et al. (1993) reported a decrease in the contraction times of rat medial gastrocnemius muscle and suggested that this could be due to an increased proportion of fast-twitch motoneurones reinnervating the muscle. Myles (1990) found that the isometric twitch contraction time of rat extensor digitorum longus, at 150 days after nerve-to-nerve suture and muscle graft, was slower than control values and she suggested that this could be explained by the reinnervation of this fast-twitch muscle with slow motoneurones. However, after 300 days there was no significant difference between the twitch contraction times of control and nerve-to-nerve suture groups which would suggest that after nerve-to-nerve suture, the types of muscle fibre present in the extensor digitorum longus were no different from control muscle. Therefore, the increase in contraction time could be explained by a reduced conduction velocity of those nerve fibres newly innervating the muscle. Myles (1990) said that this was a possibility

considering that slow motoneurones are smaller in diameter than fast motoneurones and that fibre size is reduced after nerve regeneration. Bàràny and Close (1971) found no significant difference in the duration of twitch contraction of normal rat soleus muscle and self-reinnervated soleus muscle. However, after cross-innervation of the nerve to the soleus with the nerve to the extensor digitorum longus, the duration of twitch contraction of the soleus muscle was reduced significantly. In the experiments presented here, no significant change in the duration of contraction of the soleus muscle was found after nerve injury and repair. These results suggest that, the degree of cross-innervation may have been insufficient to cause a significant change in the duration of twitch contraction. Gillespie *et al.* (1986) also showed no difference in the speed of contraction of reinnervated soleus muscle in rats and they suggested that this might be due to resistance of soleus muscle fibres to conversion. Lewis and Chamberlain noticed a larger than expected proportion of slow muscle fibres in reinnervated rat soleus muscle and suggested that this was due to the 'dedifferentiation' of motoneurones after axotomy and their failure to become 'redifferentiated' when they reinnervated a slow muscle. Consequently, the change in the properties of the fast motoneurones make them unable to convert slow muscle fibres to fast (see chapter 6.1.14).

Time to half peak tension.

Table 6.25 shows the values of the mean, standard deviation, standard error of the mean and the coefficient of variation of the time to half peak tension for non-diabetic animals and table 6.26 shows the equivalent values for the diabetic animals. Significance tests showed no difference between the diabetic and non-diabetic animals ($p > 0.05$) but in each case the time to half peak tension was slightly longer in the diabetic animals than the

non-diabetic animals. As in the findings for mean time to half peak tension, a comparison of the means of the time to peak tension between like groups of non-diabetic and diabetic animals (tables 6.23 and 6.24, respectively) shows that the time to peak tension was also slightly longer in the diabetic animals than the non-diabetic animals. The amount by which the mean time to peak tension of the diabetic animals differed from that of the non-diabetic animals, was very similar to the amount by which the time to half peak tension of the diabetic animals exceeded that of the non-diabetic animals. These results suggest that the shape of the isometric myogram up to the time when maximum twitch tension was obtained was similar for diabetic and non-diabetic animals. The results also suggest that the slightly longer time taken by the diabetic soleus muscle to reach half peak and peak twitch tension was due to slower conduction by the diabetic nerve and not because of a decrease in the speed of contraction of diabetic muscle. In other words, the decrease in conduction velocity as a result of the diabetic state caused a delay in the initiation of muscle contraction from the time of stimulating the sciatic nerve. The difference between non-diabetic and diabetic animals in the time taken to attain maximal twitch contraction was least in the muscle graft groups. In fact, the mean time to peak tension of the diabetic muscle graft group was less than that of the non-diabetic muscle graft group. This finding can be explained by the fact that the conduction velocity of nerve after muscle grafting is very slow (see chapter 4) and, therefore, the initial delay from the time of applying the stimulus to the initiation of contraction is equally slow for both non-diabetic and diabetic animals.

Duration of the twitch contraction.

The mean durations of muscle twitch as for each group of animals are diplayed in figure 6.29 and 6.30 for non-diabetic and diabetic animals, respectively. Myles (1990) found that the duration of twitch of rat extensor digitorum longus was approximately 100 ms. In the present experiments, the mean duration of twitch contraction of the soleus muscle in the non-diabetic control group was 260 ms. The longer duration of twitch contraction of soleus as compared to extensor digitorum longus of Myles (1990) is to be expected from the differences in muscle type. There was no significant difference in the duration of muscle twitch in like groups of non-diabetic and diabetic animals ($p > 0.05$). Nor was there any significant difference in any of the different groups of animals ($p > 0.05$).

6.4.3 Recovery of Muscle Twitch (direct stimulation).

One of the reasons for stimulating the soleus muscle directly was to show any differences in maximal contraction of muscle as a result of nerve injury and regeneration without considering the combined changes in the nerve and muscle as a result of injury and repair. The voltage required to overcome the impedance of the muscle sheath and other interstitial structures and bring about maximal muscle contraction results in a massive uncontrolled response that is not comparable to the controlled isometric twitch contraction produced when a muscle nerve is stimulated. Also, the contraction of all muscle fibres within the muscle cannot be ensured. Nevertheless, it was still possible to compare the findings of one experimental group with another and observe the differences between different repair groups and between diabetic and non-diabetic animals.

Hems (1993) found difficulty in activating all of the muscle fibres within the extensor digitorum longus of his rabbits, when this muscle was stimulated directly. The small values of tension produced in the rat soleus muscle by direct stimulation in the present experiments (see tables 6.11 and 6.12 for non-diabetic and diabetic animals, respectively) might suggest that in these experiments not all of the fibres had been activated. However, Nyström (1968) studying post-tetanic potentiation commented that in none of the muscles: gastrocnemius, soleus or extensor carpi radialis of kittens, was the tension of single non-potentiated twitches produced by direct stimulation of the muscle, equal to the tension produced by indirect motor nerve stimulation. In fact, the values of tension produced in the soleus muscle by direct stimulation were 0% to 32% smaller than the tensions produced by indirect stimulation. This is not surprising if the spread of contraction across the muscle is considered. Indirect stimulation causes simultaneous contraction of motor units and the summation of the tensions produced by individual motor units approximates to the total tension of that muscle (Close, 1967). The spread of contraction after direct stimulation is such that the tension produced at any one time is likely to be sub-maximal. The findings of the direct stimulation experiments in this thesis were similar to those of Nyström (1968). The mean twitch tension of the non-diabetic control group that was produced by direct stimulation of the soleus muscle was 51.98% of the mean twitch tension produced by indirect stimulation. The equivalent value for the diabetic control group was 66.12% of the mean twitch tension produced by indirect stimulation.

Interestingly, the values of mean time to peak tension and duration of muscle contraction of the non-diabetic animals (tables 6.36 and 6.38, respectively) tended to be shorter in the operated groups than the means of the control group, although no significant difference was found between

any of the means ($p > 0.05$). The values of mean time to peak tension and the duration of contraction of the diabetic animals (tables 6.37 and 6.39, respectively) followed a similar pattern to that of the non-diabetic animals. The operated groups tended to have smaller mean times of contraction and duration of muscle twitch than the control group. The shorter times to peak tension of the nerve-to-nerve suture, nerve graft, and muscle graft groups as compared to the control and crush groups, in both diabetic and non-diabetic animals, may be due to the size of muscle. The mean mass of the soleus muscle of the operated groups of animals were smaller than the mean mass of the control groups (see chapter 6.3.6). Direct stimulation of a muscle that causes its contraction involves depolarization of the membranes and release of Ca^{2+} from the sarcoplasmic reticulum. If the spread of depolarizing current across a muscle takes longer in bigger muscles, the larger the muscle, the more time will have to elapse before maximal contraction is attained. Massive uncontrolled release of Ca^{2+} as a result of direct stimulation may be the reason behind the increased duration of twitch in the larger control muscles. This is because relaxation is brought about by uptake of Ca^{2+} by the sarcoplasmic reticulum. The removal of Ca^{2+} such that the concentration falls below threshold levels for contraction may take longer in larger muscles that produce greater tensions.

CHAPTER 7

Assessment of Proprioceptive Recovery

7.1 Introduction.

7.1.1 Aims.

Patients with otherwise good recovery of motor function and sensation after peripheral nerve injury and repair frequently complain of poor motor coordination. It is for this reason that an investigation into the return and recovery of the monosynaptic component of the stretch reflex was undertaken. Small amplitude vibration of any muscle causes excitation of the primary endings of Ia afferent nerve fibres which terminate on the intrafusal fibres of muscle spindles and, via a monosynaptic reflex arc, causes reflex contraction of the extrafusal muscle fibres of the same muscle (Matthews, 1966). Using the methods of Matthews (1966) to investigate the tension produced in mammalian skeletal muscle as a result of initiating a reflex contraction in that muscle, the return and recovery of the stretch reflex in the soleus muscle of rats were studied after different methods of nerve injury and repair, in non-diabetic and diabetic animals.

7.1.2 The Anatomy of the Muscle Spindle.

The muscle spindle is composed of specialized skeletal muscle cells which are contained within a fluid-filled capsule and are thus called intra-fusal fibres.

Onanoff (1890) observed the degeneration of the intra-fusal fibres of muscle spindles after section of the dorsal root in the dog and this finding suggested that the function of the muscle spindle was that of a sense organ. Ruffini (1898) and Sherrington (1894) went on to confirm the sense organ

theory by demonstrating spindle innervation: Ruffini by using gold chloride stains and Sherrington by nerve section and by tracing large fibres to the spindle.

Up to twelve intrafusal muscle fibres are contained within a fluid-filled lamellated capsule. The fibres run parallel to each other and also to the extra-fusal fibres. The intrafusal muscle fibres were thought to be of two distinct types: the nuclear-bag and the nuclear-chain fibres. It is now known that the nuclear-bag fibres can be divided into bag_1 and bag_2 types (Barker & Gidumal, 1961).

The nuclear-bag fibres were first described by Barker (1948) and contain nuclei in clusters in the equatorial region of the spindle. Two different types of fibre were first observed by Cooper and Daniel (1956); chain fibres are fast contracting and have nuclei arranged in chains. The number of chain fibres predominates over the number of bag fibres within each spindle. An intermediate type of fibre was noticed by Barker and Gidumal (1961) that was, morphologically, a small bag fibre but with an ultrastructure like that of a chain fibre.

7.1.3 Nerve Supply of Intrafusal Fibres.

The nerve endings terminating on intrafusal fibres were first described by Ruffini in 1898. He described three types of nerve ending:

(1) Annulospiral ending supplied by a nerve fibre of large diameter;

(2) Flower-spray ending supplied by a nerve fibre of smaller diameter;

(3) Plate ending supplied by nerve fibres of very small diameter. The endings were distributed in the polar regions of the spindle.

To this day the above terminology is used although this simple description has been much elaborated upon. The sensory innervation is now understood to consist of primary endings which arise from Ia afferent

nerve fibres and terminate on the central portion of all intrafusal fibres. The terminal branches of the ending each give rise to one, or occasionally two systems of annulospiral terminals. The usual fibre-to-ending ratio being 1:1 (Banks & Barker,1988). The secondary endings arise from the smaller group II afferent nerve fibres and can take the form of, the more common, annulospiral and also the irregular flower-spray type ending (Barker, 1948). Secondary endings terminate at none, one or both sides of the primary region. Secondary endings are almost exclusively found on chain fibres.

There is also more than one type of motor fibre innervating muscle spindles. γ motor neurons only supply the intra-fusal fibres of muscle spindles and are thus called fusimotor. In 1962 Boyd and Eccles differentiated between two types of γ fibre: γ_1 and γ_2; the former supplying the bag fibres with plate endings and the latter supplying the chain fibres with diffuse endings. The subtypes of γ motor innervation of muscle spindles have also been categorized depending on their specific effects on the discharge of afferent nerve fibres. Dynamic γ neurons have an excitatory effect on Ia afferent discharge during changes in length of the muscle but rarely influence the secondary endings of intra-fusal muscle fibres. In contrast, static γ neurons decrease the dynamic responsiveness of the primary and secondary endings of intrafusal muscle fibres and increase the response of both types of afferent ending to static stimuli. The dynamic index is the value given to the fall in afferent fibre discharge in 0.5 s after maximum stretch has been attained. Stimulation of the dynamic fibre increases this index, whereas stimulation of the static fibre decreases the dynamic index. It is now thought that the dynamic γ nerve fibres produce their effects on bag_1 muscle spindle fibres and the static γ produce their effects on bag_2 and chain fibres, although static γ nerve fibres may well supply bag_1 muscle spindle fibres also (Matthews, 1964). β efferent nerve

fibres supply both extrafusal and intrafusal muscle spindle fibres simultaneously and are thus called skeleto-fusimotor. These β nerve fibres are also divided into static and dynamic types.

7.1.4. Function of the Muscle Spindle.

The muscle spindle forms the receptor for proprioceptive monitoring of muscle length and rate of change of length. Stretching of the whole muscle causes simultaneous stretching of the intrafusal muscle fibres. This in turn causes distortion of the sensory terminals, depolarization of the terminals and initiation of action potentials in the afferent fibres. The stimulus to the muscle can be divided into static and dynamic components. Intrafusal fibres respond to these components i.e. change of length of muscle (static component) and rate of change of length of muscle (dynamic component). It is known that both primary and secondary endings respond to the static component while primary endings are markedly more sensitive to the dynamic component. The reason for this difference is thought to be based on the mechanical properties of the intrafusal fibres. The tissue of the central area of bag fibres is more viscous than the poles of the fibre, which results in the relationship between the load on the fibre and its extension not being linear; the central area of the bag fibre has been described in engineering terms as behaving rather like a 'dash-pot'. Hence, primary endings terminating on central regions of bag fibres show greatest sensitivity to the dynamic component of stretch. Secondary endings terminating on either side of the primary region respond to the static component of stretch. This is thought to be due to the more elastic properties of the fibre in these regions which cause it to behave rather like a spring so that the relationship between load and extension is linear (Hooke's Law).

The simple view of the stretch or myotatic reflex is that excitation of the Ia fibres causes reflex contraction of homonymous muscles (autogenetic excitation) and synergistic muscles (heterogenetic excitation) *via* monosynaptic connections, and inhibition of the antagonistic muscles *via* disynaptic connections involving inhibitory interneurons (reciprocal inhibition). In addition, there is recurrent inhibition where excitation of a spinal motoneuron stimulates a Renshaw cell. Excitation of the Renshaw cell causes inhibition of spinal motoneurons including the one which elicited a response in that cell.

The whole picture of the stretch reflex is far more complicated, with polysynaptic pathways also affecting it. However, the aims of the experiments of this thesis were to investigate the return of the monosynaptic component of the stretch reflex which elicits muscle contraction in the same muscle as that stimulated by small vibratory stretch, after different types of nerve injury and repair.

The presence of the inverse myotatic reflex may also be important in the work presented here (see chapter 7.4). This reflex has the reverse effects to the myotatic reflex (i.e. homonymous and synergistic muscles are inhibited and antagonistic muscles are excited) and is thought to be ellicited by excitation of Ib afferent fibres which serve Golgi tendon organs.

Group II afferents may also contribute to the myotatic reflex. Matthews (1964) found that if the Ia fibres were saturated by tonic vibration stimuli, a stretch reflex could still be induced and this may have been due to excitation of group II afferents.

7.1.5 Control of Reflex Action.

A stretch or myotatic reflex occurs when limbs or muscles resist an opposing force by muscular contraction, for example: if an extended limb is

forced into a flexed position, the extensor muscles will contract to oppose the force being applied. Stretch reflexes are strongest in physiological extensors and are greatest in decerebrate animals (Sherrington, 1909). The reason for the stretch reflex being at its strongest in the decerebrate animal is associated with the loss of the inhibitory effects of descending neuronal pathways on the reflex response. The transection used classically to achieve decerebration and produce extensor rigidity is that between the inferior and superior colliculi of the mid-brain. After transection, the descending excitatory effects from the cortex on the fibres of the medial reticular extensor inhibitory area, are lost. In contrast, most of the excitation of the lateral reticular extensor facilitatory areas is from ascending pathways and not from the cortex. In effect, transection of the mid-brain between the superior and inferior colliculi results in maintenance of most of the excitatory effects from the lateral reticular extensor facilitatory areas and loss of the inhibitory effects from the medial reticular extensor inhibitory area. If the excitatory effects from the lateral reticular extensor facilitatory areas are lost (e.g. if the transection is made too far caudally so that the excitatory effects of the ascending pathways on the facilitatory areas are destroyed), extensor rigidity is absent. Excitation of α and γ motoneurons also comes from the lateral vestibular nucleus (Deiter's nucleus) and in the control situation, the Purkinje cells of the anterior lobe of the cerebellum directly inhibit Deiter's nucleus and the fastigial nucleus (which has excitatory effects on Deiter's nucleus) such that the excitation of Deiter's nucleus is not enough to produce decerebrate rigidity. If however, a second incision is made across the anterior lobe of the cerebellum, and the blood supply to the rostral mesencephalon is shut off by ligation of the basilar and carotid arteries, the excitation of Deiter's nucleus is such that extensor rigidity occurs whether or not the ascending afferent pathways are intact (Pollock & Davis, 1923).

7.1.6 Effects of Nerve Injury on Muscle Spindle Reinnervation.

After denervation of skeletal muscle, the atrophy of intrafusal muscle fibres was observed by Onanoff (1890) and more recently by Myles (1992) and by Quick & Rogers (1983). This atrophy is reversible on reinnervation of the spindles although Myles (1992) found it more difficult to differentiate between chain and bag fibres. Other authors have found that reinnervated muscle spindles are morphologically recognizable on histological examination but still abnormal to some degree (Quick & Rogers, 1983). Barker, Scott and Stacey (1985) found that the reinnervation of cat muscle spindles after nerve crush sometimes resulted in hyperinnervation of intrafusal muscle fibres and primary afferent endings that were shorter than normal. They also found evidence of some secondary afferents having regenerated through the primary region of the muscle spindle to reach the opposite pole which was lacking in secondary endings. Barker, Scott and Stacey (1986) investigated the reinnervation of cat muscle spindles after different periods of denervation. They found after long-term denervation of muscle spindles (produced by repetitive crush injury) and subsequent reinnervation that, although the afferent fibres reinnervating muscle spindles were abnormal in terms of the primary endings being shorter and having fewer transverse bands, the responses of the endings to ramp-and-hold stretch were relatively unimpaired. Therefore, it seems that although regenerated and reinnervated muscle spindles may have abnormal appearances, they can still respond to their proper stimuli.

The normal functioning of muscle spindles may also be dependent on the recovery of appropriate afferent and efferent connections and this is more likely to occur after nerve crush injury, when regenerating axons can grow down the original endoneurial tubes and reform the same peripheral

connections as before. Brown and Butler (1976) studied the regeneration of the nerves to cat peroneus longus and tenuissimus muscles after nerve crush, and transection and suture. They found normal reinnervation of muscle spindles with primary and secondary afferent fibres, and static and dynamic efferent fibres, although some abnormal innervation was found. These authors said that afferent and efferent fibres showed preference for their original sites during reinnervation. Hyde and Scott (1983) found that the recovery of muscle spindles after nerve crush in cats was very good. The common peroneal nerve was crushed and after 118 days only 11% of spindle afferents behaved abnormally to ramp and hold stretching of muscle spindles. However, after transection and suture of the common peroneal nerve in cats, Banks, Barker and Brown (1985) found that the recovery of functionally identifiable muscle spindle afferents was only 25% of normal and the reinnervation of Golgi tendon organs was only 45% of normal. These aforementioned authors also investigated the reinnervation of muscle spindles after "short length nerve autografts" which are equivalent to the nerve grafts performed on the rats used in the experiments presented in this thesis. After this type of repair, with two suture lines, the reinnervation of muscle spindles with functionally identifiable spindle afferents was only 10% of normal. The authors suggested that incorrect connections had been made and this would probably result in abnormal reflex action. Myles (1992) also suggested, from her work on the reinnervation of muscle spindles in rats, that it is likely that proprioceptive reflexes would be adversely affected through inappropriate reinnervation of muscle spindles after muscle grafting.

Banks and Barker (1989) investigated the specificity of afferents reinnervating cat muscle spindles after nerve transection and repair. They performed cross-union experiments whereby foreign afferents including Ib

afferents were allowed to reinnervate muscle spindles in the absence of Ia or II afferents. Interestingly, they found that Ib afferents could reinnervate spindles in place of the original Ia and II afferents and respond to stretch in a similar way to Ia and II afferents. In fact, 21% of spindles in peroneus brevis were reinnervated by Ia afferents and 20% by Ib afferents after complete section and regeneration of the peroneal nerve. Some cutaneous afferents were also found to reinnervate spindles, where Ia afferents once served, and give Ia type responses. Although efferent fibres appeared to regenerate more quickly than afferent fibres, they were only rarely found to enter muscle spindles via afferent endoneurial tubes. This result agrees with the findings of Brushart and Seiler (1987) who found that regenerating nerve fibres demonstrate afferent and efferent specificity. Banks and Barker (1989) have stated that efferent-afferent reconnection probably does not occur. The potential effect of inappropriate reconnection on the stretch reflex is discussed in chapter 7.4.

7.2 Materials and Methods.

7.2.1 Anaesthesia.

150 days after the initial nerve repair procedures (chapter 2.6), each animal was anaesthetized. The first 10 animals were anaesthetized by the inhalational method of anaesthesia described in chapter 2.3.2 and were subsequently decerebrated (see below). Owing to the lack of success in achieving hyperreflexia in the decerebrate animals, an operation which is notoriously difficult to perform in rats (personal discussion with Barker, 1991), the animals used subsequently were anaesthetized using an intra-muscular injection of neuroleptanalgesic and benzodiazepine (chapter 2.3.1) and were not decerebrated. The disadvantages of using the latter type of anaesthesia are discussed in chapter 7.4.

The effects of inter-collicular decerebration in producing a state of hyperreflexia are explained in chapter 7.1.5. The advantage of anaesthetizing the rats with a gaseous mixture of halothane, nitrous oxide and oxygen, in experiments such as these, is that the effects of the anaesthetic 'wear off' very rapidly after its withdrawal and, therefore, do not interfere with the reflex action. In the animals used in the decerebration experiments, ventilation of the rats' lungs with oxygen was continued after the withdrawal of anaesthetic. This ensured a continuous supply of oxygen to the lungs in order to maintain consistent levels of blood gases (although the levels were not monitored).

Every animal used in the experiments described here, was kept warm on an electrically heated blanket and an injection of 10 ml of saline at 37^{o}C

was made subcutaneously at the scruff of the neck. The purpose of the saline was to replace any fluid that may have been lost through evaporation at the operation site.The problems associated with using a combination of inhalational anaesthesia and decerebration included the loss of large amounts of fluid. Although subcutaneous injections of saline were given, it was unlikely that the fluid lost through evaporation from the site of the tracheostomy and importantly, the loss of blood from the cerebral vessels during the process of decerebration (see below), were replaced. Although animals were warmed by an electrically heated blanket while operating, the extended duration of the preparatory procedures for those rats who received inhalational anaesthetic and were decerebrated, thereby losing hypothalamic thermoregulatory control, was such that the core temperature of these rats fell to variable degrees during the course of the experiment. The evaporation of water from the sites of operation, particularly from the head, doubtlessly contributed to the cooling of the body temperature of each animal. Out of the 10 animals that were decerebrated, only 5 survived the experiment and only 1 of these showed the classic signs of decerebrate rigidity. A reflex tension in the soleus muscle (see below) was not produced in any of the decerebrate animals. It is for these reasons that the alternative, intra-muscular injection of neuroleptanalgesic and benzodiazepine, was used in animals used in the subsequent experiments.

7.2.2 Decerebration.

The rats to be decerebrated were anaesthetized as described in chapter 2.3.2. After attaching the animal to the ventilator, the carotid arteries, situated on either side of the trachea, were exposed. Liga-clips were applied to ligate the vessels using a liga-clip applicator. The animal was turned onto its abdomen and electrically operated clippers were used to shave the

animal's head. An incision was made through the skin, extending from the posterior occipital crest to the nose. The skin was retracted and the temporalis muscles were removed using a scalpel. The exposed bone was removed gradually using an electric dental drill until dura mater was exposed. Bone rongeurs could then used to remove bone, piecemeal, until the cerebellum and cerebral hemispheres were visible. Severance of the superior sagital sinus resulted in some bleeding. Bone wax (Ethicon Ltd, U.K.) was used to plug the bone and arrest bleeding when possible. (N.B. personal discussion with Banks and Barker revealed that the bleeding from the venous sinuses was an inevitable and unpreventable consequence of performing decerebrations. They found that if the bleeding was allowed to continue, the flow decreased over time.) A Sonocut Maxi (Codman and Shurtleff Inc, Randolph, Massachussettes, U.S.A.) ultrasonic neurosurgical dissector was used to disrupt brain tissue ultrasonically, while simultaneously irrigating the area with saline and removing debris by suction. This machine enabled very careful and gradual removal of brain tissue. The region of the inferior colliculi is obscured in the rat by the occipital lobes, however, using an atlas of rat neuroanatomy as a guide, the level at which the decerebration should be made could be judged quite accurately. Commencing with removal of tissue from the frontal lobes, brain was removed gradually and in a caudal direction, to the level between superior and inferior colliculi.

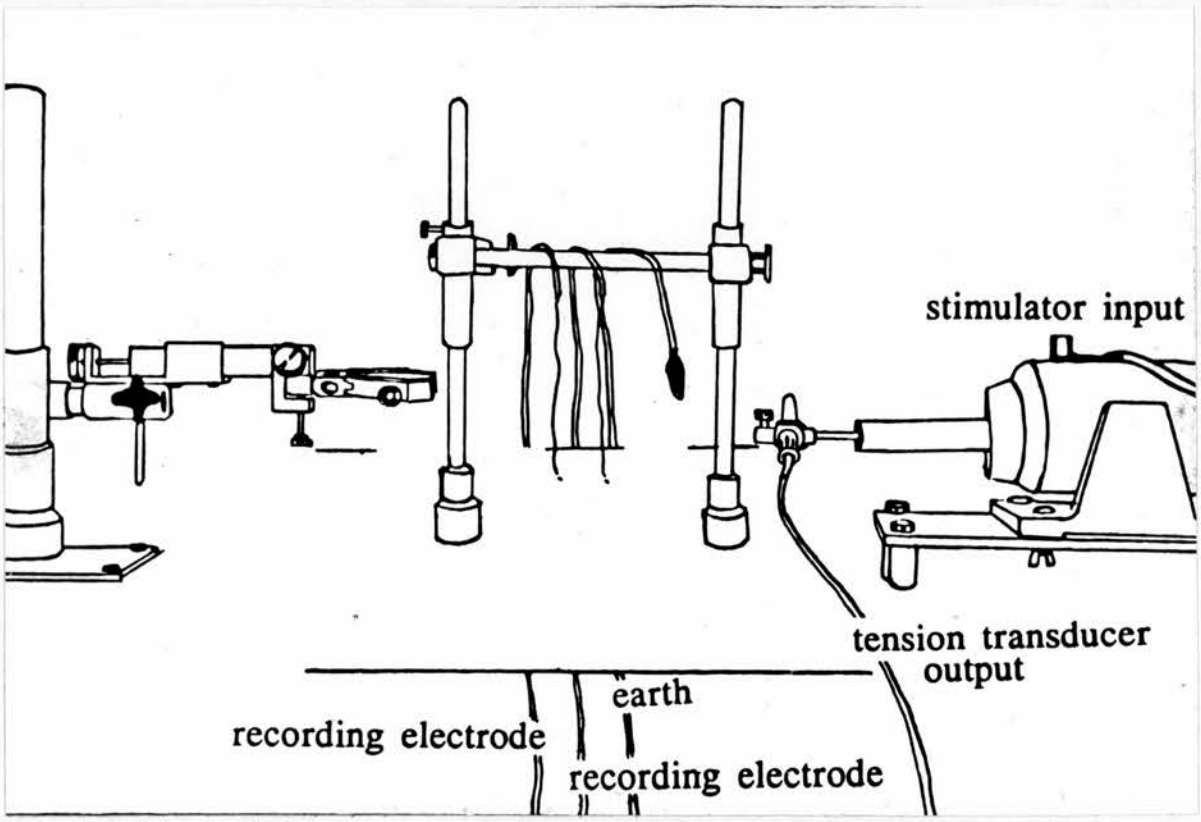
7.2.3 Preparation of Experimental Animals.

The left sciatic and common peroneal nerves were exposed as described in chapter 2.4. and the peroneal and sural nerves were divided (chapter 6.2.1). A second incision was made on the medial aspect of the left thigh, extending from the pubic bone to the anterior superior iliac crest. The

skin was retracted to expose the femoral vessels. Using an operating microscope (Weck Fibermatic 0902A1, Long Island City, New York) to aid vision, the femoral nerve situated laterally to the femoral vessels was exposed and divided. The skin of both wounds were sutured using uninterrupted 3/0 Vicryl sutures (Ethicon Ltd, Edinburgh, U.K.). The reason for closure of the wounds was to prevent excessive evaporation and fluid loss.

The soleus muscle was exposed in the way described in chapter 6.2.2. The rat was moved to a table in a Faraday cage. The left leg was clamped rigidly, almost perpendicular to the table, by two clamps in the same way as described in chapter 6.2.3. The distal end of the tendo calcaneus was attached by a sharp tendon hook to an inextensible steel rod, devised for the purpose of the experiments presented here (produced by staff of the mechanical workshop, The Royal Dick Veterinary College, Edinburgh University), whose proximal end was rigidly attached to the beam of an isometric force transducer (Lectromed 4150). The force transducer was mounted at the end of the moving element of a V201 vibrator (Ling Dynamic Systems Ltd, Royston, U.K.). The moving element of the vibrator was arranged so that its movement was parallel to the table and in the horizontal plane of the muscle fibres. Small amplitude sinusoidal vibration was delivered to the soleus muscle using this apparatus. The arrangement of the mechanical apparatus is displayed in figure 7.1.

Figure 7.1 A diagram of the arrangement of the mechanical apparatus used in the reflex tension experiments



7.2.4 Recording Reflex Tension.

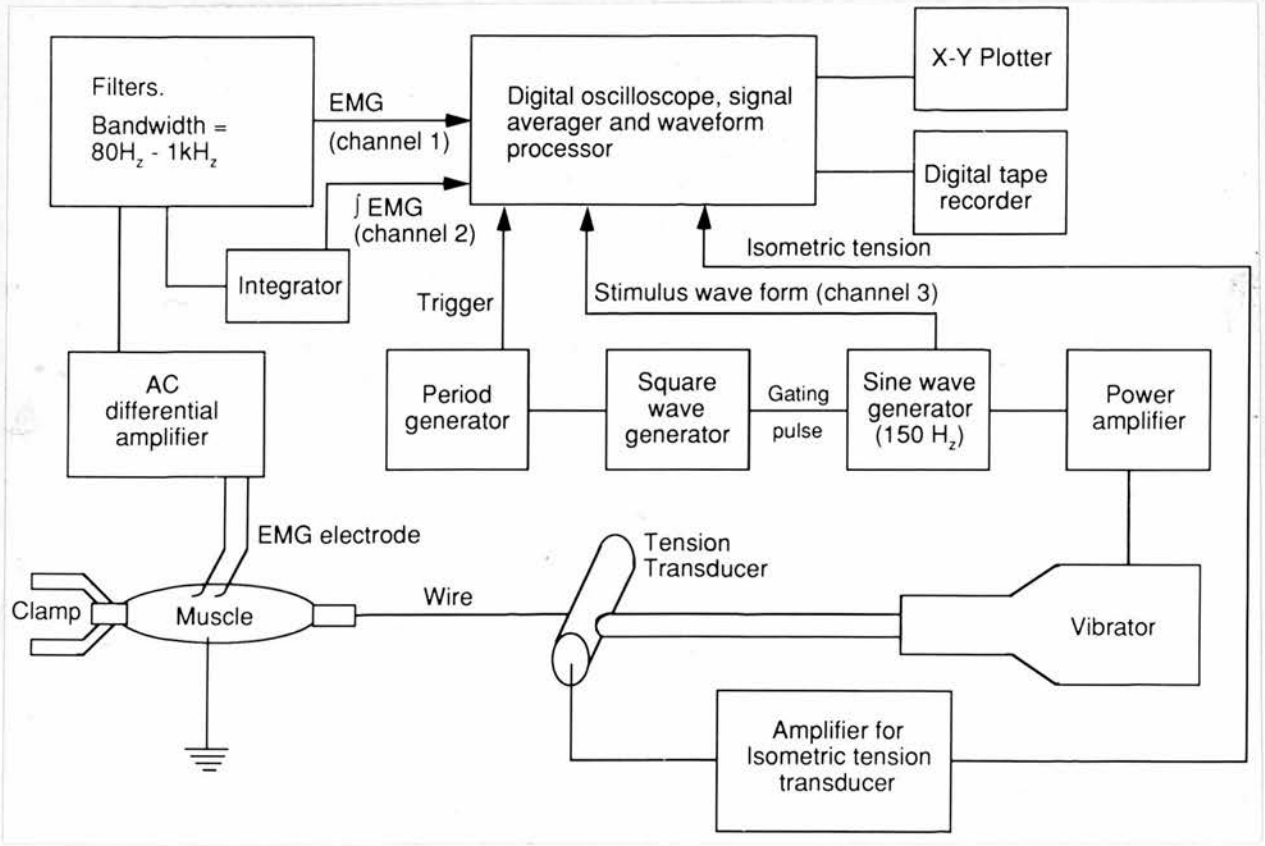
A diagram of the electrophysiological apparatus is shown in figure 7.2. A Neurolog, NL 304 period generator (Digitimer Ltd, Welwyn Garden City, U.K.) was used to trigger a Gould 7074 digital storage oscilloscope and 704 waveform processor (Gould Electronics Ltd, Ilford, U.K.). the output of the period generator was, simultaneously, passed to a Neurolog, NL 403 delay-width module (Digitimer Ltd, Welwyn Garden City, U.K.). The output of the period generator module could be controlled, in off/reset mode, to deliver the stimulus on demand. The delay-width module was arranged to produce a 100 ms delay after the triggering pulse followed by a 500 ms

square wave stimulus at 1 V. The delay was used simply to allow a baseline to be recorded on the oscilloscope. The square wave output produced by the Neurolog modules was passed to a Thandar TG501 Function generator (RS Components Ltd, Corby, U.K.) where it provided a gated input. The function generator was used to produce an electrical sine wave output, between +1 and -1 V peak-to-peak amplitude in to a 50 Ω load. The frequency of the sine wave output could be varied at the function generator. The output of the function generator was sent to an AC power amplifier where the amplitude of the vibration could be controlled. A second and simultaneous output from the function generator was sent to a y-input of the oscilloscope where a refenerence trace for the stimulus was displayed on the oscilloscope screen. The amplitude of the stimulus was varied at the power amplifier from which the output was used to drive the vibrator (see above). A calibration graph of the power amplifier had been constructed by plotting the numerical settings of the amplifier against the peak-to-peak amplitude of the vibration. The output of the force transducer was sent to a Neurolog NL107 DC amplifier (Digitimer Ltd, Welwyn Garden City, U.K.) from which the signal was sent to a y-input of the oscilloscope. The baseline could be adjusted using the DC offset facility of the amplifier. Using the waveform processing facility of the oscilloscope, the deflection produced on the oscilloscope screen as a result of the force developed in the muscle was measured. The force transducer was calibrated in the same way as the tension transducer used in the experiments described in chapter 6 (see chapter 6.2.5). Appendix 18 shows the calibration curve of the force transducer (Lectromed 4150).

The soleus muscle was vibrated at a range of amplitudes: from 50 μm to 700 μm. The frequency of vibration was also altered; frequencies of 100 Hz, 150 Hz, 200Hz, 250 Hz, 300 Hz were used. The muscle was allowed

to rest for 3 minutes between vibrations, however, in many cases the muscle fatigued before all the the measurements could be completed. The first frequency to be used was 150 Hz so that a complete set of results were obtained for this frequency.

Figure 7.2 A diagram of the electrophysiological apparatus used in the experiments for recording reflex tension in the soleus muscle



7.2.5 Recording of the E.M.G.

For some frequencies and amplitudes very small tensions were obtained but the existence of an E.M.G. indicated that there was muscular activity. A diagram of the electrophysiological apparatus used for recording E.M.G. is shown in figure 7.2. Two low impedance, silver wire electrodes were inserted into the soleus muscle; one was placed adjacent to the soleus/tendocalcaneous junction and the other was inserted into the belly of the muscle, 2 cm proximal to the first electrode. The electrodes were connected to a Neurolog NL104A AC differential amplifier and NL125 filter (Digitimer Ltd, Welwyn Garden City, U.K.). The filter was set at a bandwidth of 80 Hz to 1 kHz. One output from the filter was connected directly to a channel on the oscilloscope to produce a display of raw E.M.G.. A second output from the filter was connected to a channel on the oscilloscope via a Neurolog Integrator (Digitimer Ltd, Welwyn Garden City, U.K.) to give a display of integrated E.M.G.. An earth electrode was inserted into the adjacent gastrocnemius muscle, and the table and Faraday cage were insulated from the floor. The preparation, table and Faraday cage were connected to ground by way of 'mains earth'.

7.3 Results.

7.3.1 Results from Reflex Tension Experiments.

The difficulty in producing decerebrate rigidity in pilot experiments has already been discussed (chapter 7.2.1). Owing to the fact that the type of anaesthesia used was a neuroleptanalgesic (see chapter 2.3), the data relating to tension, obtained by eliciting a reflex contraction in the soleus muscle of the rats and used in the experiments presented here, is only qualitative. For this reason it was considered inappropriate to apply statistical analysis to the data. If quantitative information is sought then the recordings of reflex tension should be performed in decerebrate animals; a situation when there is no influence from descending inhibitory pathways and the tensions produced are bigger and less variable. However, it was considered that it was worthwhile presenting the values of tension recorded from the soleus muscles, if only to observe the degree of variation in tension produced from animal to animal under similar anaesthesia and to demonstrate the presence or absence of an intact pathway.

The values of reflex tension obtained from each animal are presented in appendix 17. The corresponding values of isometric tetanic tension produced by each soleus muscle and the masses of the left and right muscles are also displayed in appendix 17. The results from the data of tetanic tension and the mass of the soleus muscle have been presented in chapter 6.

At the time of the final experiments (150 days after performing the initial nerve injury and repair procedures), all of the animals demonstrated an

intact toe-spreading reflex (a generalized nociceptive flexor reflex), which indicated that non-specific reinnervation had occurred. After performing the experiments to ellicit the tonic vibration reflex, each soleus muscle was stimulated to produce the maximum isometric tetanic tension in the muscle. The results are presented in chapter 6 and demonstrate that good return of force of contraction of the soleus muscle was obtained after nerve crush injury in both diabetic and non-diabetic animals. After a neurotmesis type injury and subsequent repair, the recovery of force of contraction was not as good but even after muscle grafting there was a reasonable recovery of tetanic tension (the diabetic muscle graft group produced a mean tension of 0.22 N as compared to 0.57 N produced by the diabetic control group; the non-diabetic muscle graft group produced a mean tension of 0.39 N as compared to 0.97 N of the non-diabetic control group; see tables 6.6 and 6.7). Importantly, a reflex tension was only ellicited in the soleus muscles of the control and crush groups of both the non-diabetic and diabetic populations of animals. Figure 7.3 is a plot of the amplitude of vibration (at 150Hz) against the tension produced in the soleus muscle of a non-diabetic rat from the crush group.

As described in chapter 7.2.5, the reason for recording the E.M.G. was that it provides a trace of the electrical activity of a muscle, and any reflex response in the soleus muscle that may have been too small to produce a measurable tension would have been detected. No electrical activity was recorded on the E.M.G. in any of the experiments involving rats from the nerve-to-nerve suture, nerve graft or muscle graft groups; therefore, it is unlikely that tension was produced in the soleus muscle of these animals that was not discernable with the equipment used. The values of the mean, standard deviation, standard error of the mean and the coefficient of

variation of the reflex tension that was induced in the soleus muscle of both control and crush groups are displayed in table 7.1.

Table 7.1 Values of the mean, standard deviation, standard error of the mean, and coefficient of variation of reflex tension of the soleus muscle, in the control and nerve crush groups

RAT GROUP	n	m /N	SD	SEM	c.v.
C	5	0.172	0.028	0.013	16.44
CR	5	0.163	0.022	0.010	13.72

RAT GROUP	n	m /N	SD	SEM	c.v.
DC	5	0.091	0.010	0.005	11.23
DCR	5	0.121	0.031	0.014	25.69

The standard deviation and the coefficient of variation were greatest for the diabetic nerve crush group (0.031 and 25.69%, respectively); however, much higher coefficients of variation have been observed in the experiments presented in other chapters of this thesis, for example: the coefficient of variation of recovery of receptive field area of the diabetic muscle graft group was 84.42% (see table 5.2). The standard deviation and coefficient of variation for each of the four experimental groups (see table 7.1) were such that it was considered that the effect of the anaesthetic in inhibiting the reflex tension was quite consistant from animal to animal. Although the mean reflex tension of the diabetic control group was smaller than the mean of the diabetic crush group (0.091 N measured from the diabetic control group as compared to 0.121 N measured from the diabetic crush group), the mean tetanic tension of the diabetic control group was also smaller than the mean tetanic tension of the diabetic crush group (0.724 N measured from the diabetic crush group as compared to 0.569 N

measured from the diabetic control group). Figure 7.4 shows a typical trace of raw E.M.G., integrated E.M.G., the tension produced and the reference trace of the vibratory stimulus, obtained after stimulating the soleus muscle of a rat from the non-diabetic control group; figure 7.5 shows the equivalent measurements for a rat from the non-diabetic crush group and figure 7.6 is a typical trace of raw E.M.G., integrated E.M.G., reflex tension and reference trace of the stimulus that was obtained from an animal in the non-diabetic muscle graft group. Cutting the tibial nerve and then stimulating the soleus muscle produced traces very similar to those displayed in figure 7.6.

Figure 7.3 The amplitude of vibration, applied at a frequency of 150 Hz, to the soleus muscle of a rat in the non-diabetic crush group, plotted against the tension induced in the muscle

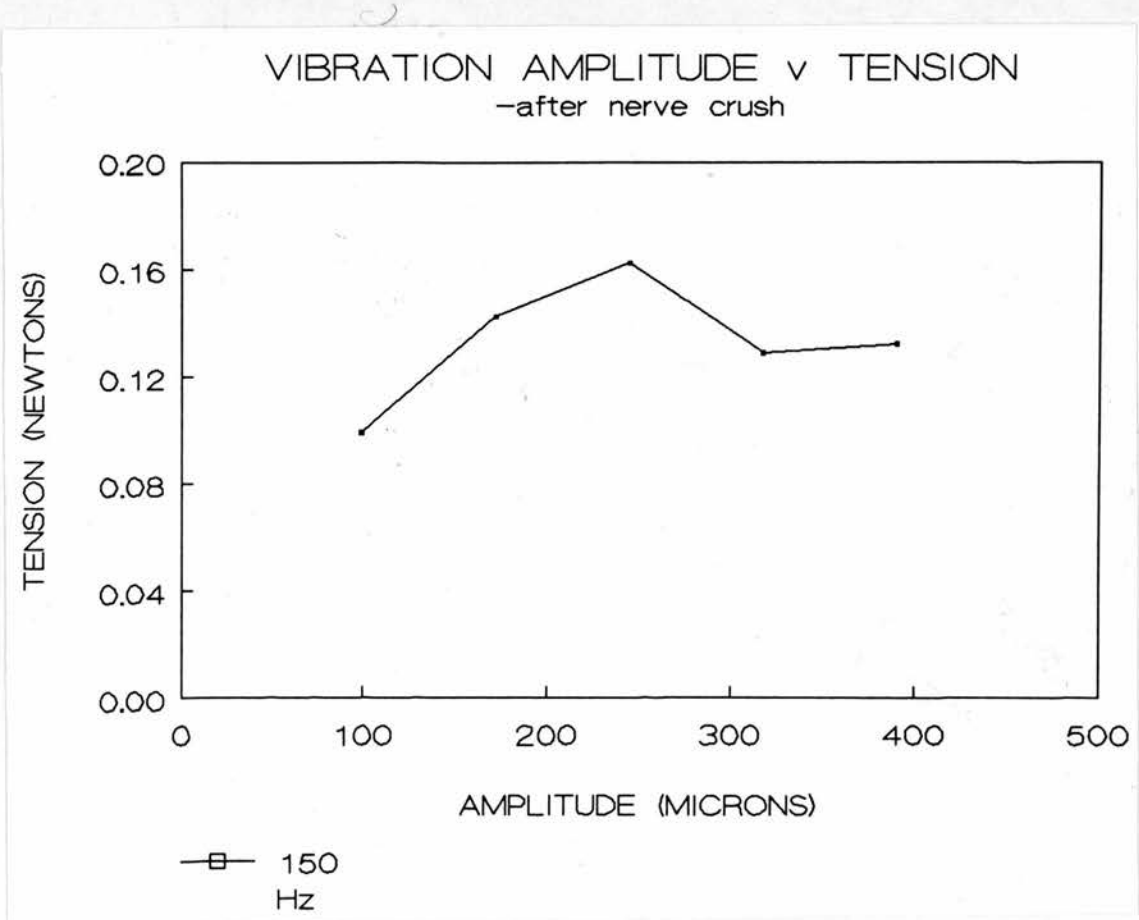


Figure 7.4 A trace of raw E.M.G., integrated E.M.G., reflex tension, and the reference trace of the vibratory stimulus obtained from a rat in the non-diabetic control group

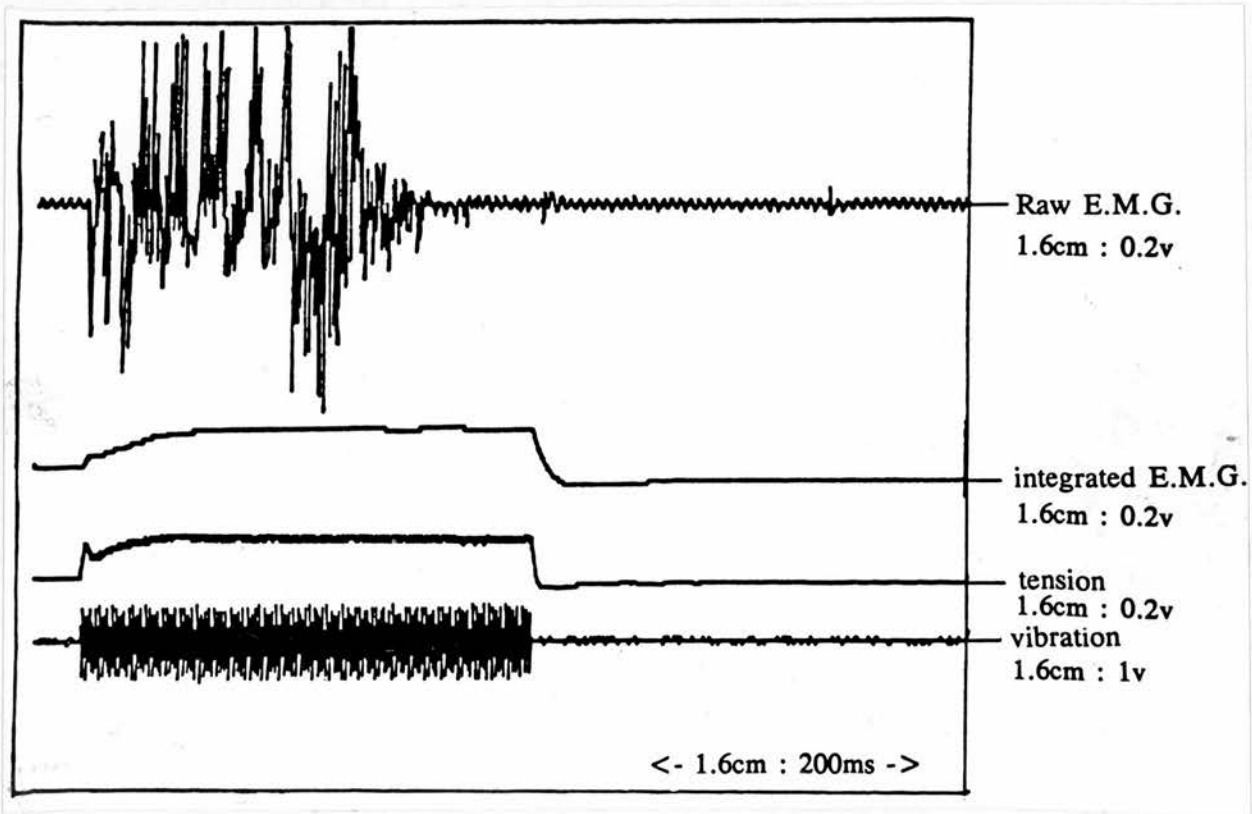


Figure 7.5 A trace of raw E.M.G., integrated E.M.G., reflex tension, and the reference trace of the vibratory stimulus obtained from a rat in the non-diabetic crush group

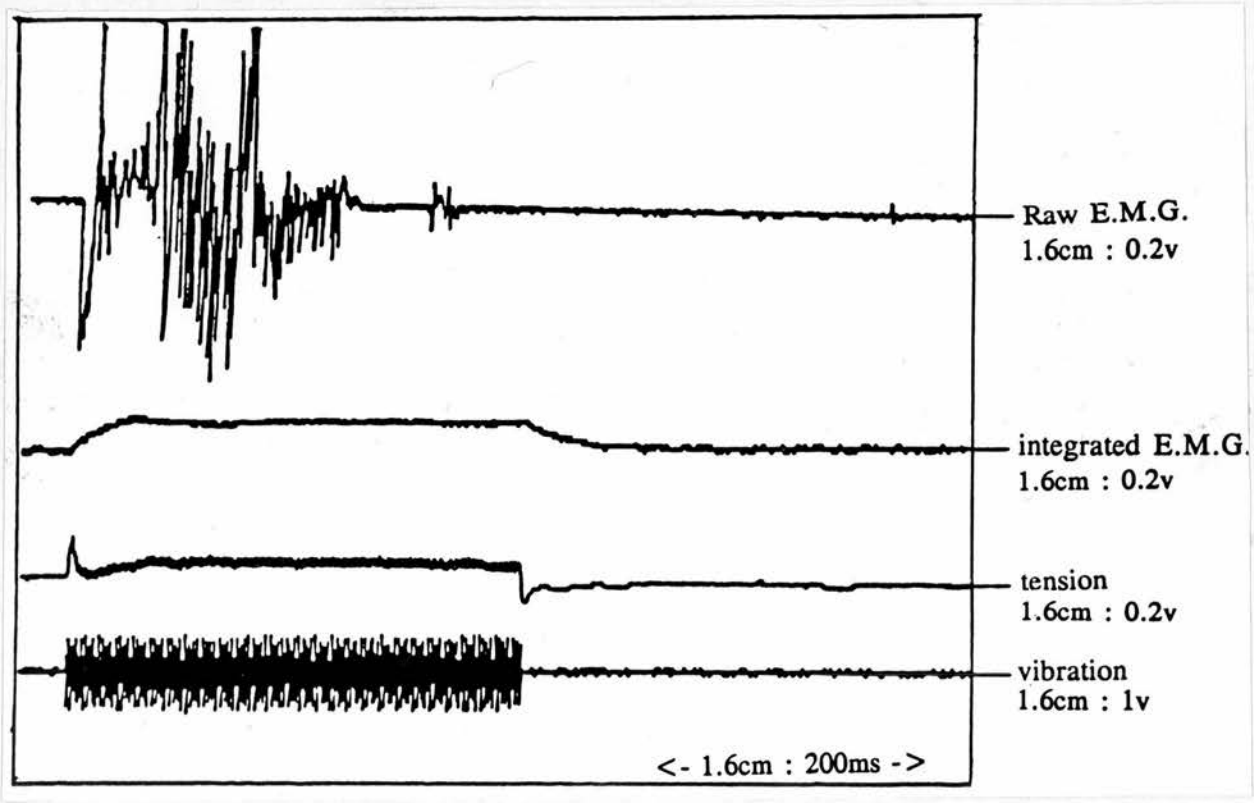
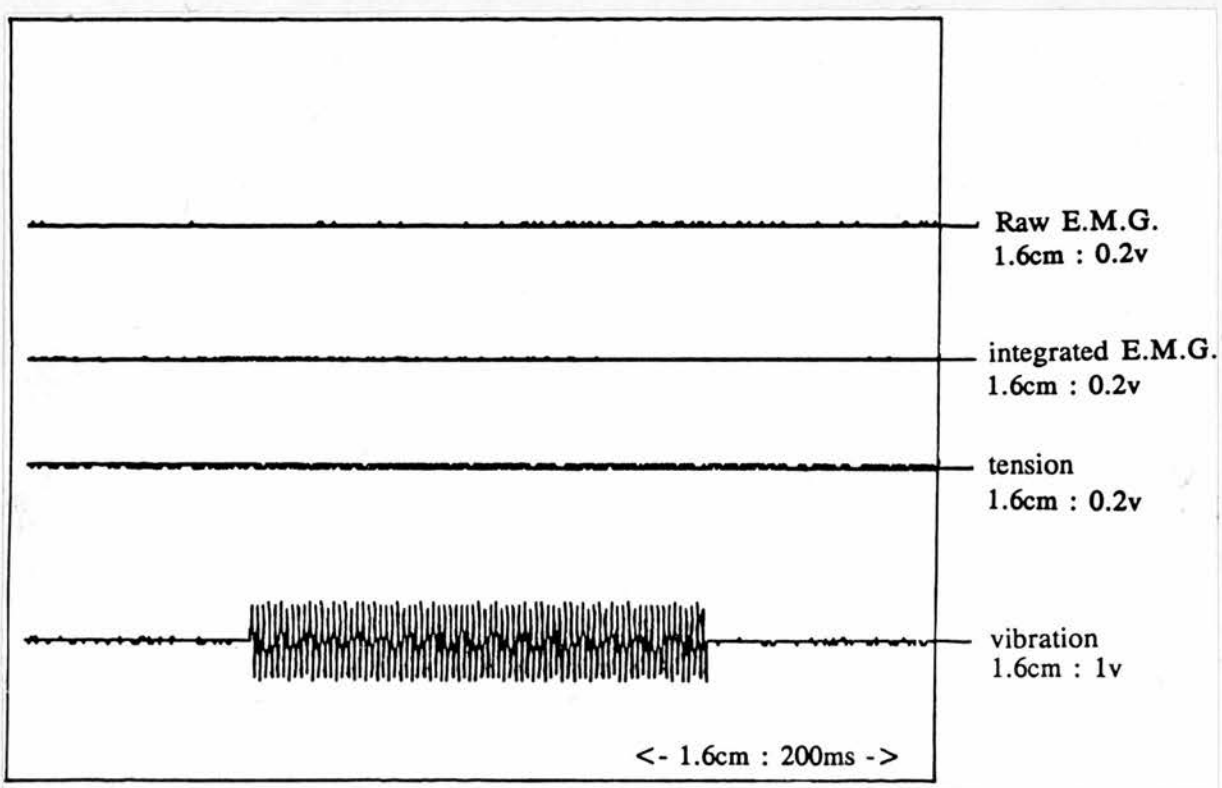


Figure 7.6 A trace of raw E.M.G., integrated E.M.G., reflex tension, and the reference trace of the vibratory stimulus obtained from a rat in the non-diabetic muscle graft group



7.4 Discussion.

The most important finding in the work presented in this chapter is that there was no apparent return of the monosynaptic stretch reflex after nerve injury of the neurotmesis type and subsequent repair. It is possible that the tensions produced by the soleus muscle after nerve transection injury were small enough to have been undetected in the experiments presented here. However, it is very unlikely that this happened because any electrical activity induced in the muscles as a result of the tonic vibration reflex would have been recorded as asn E.M.G. and no electrical activity was recorded from the soleus muscles in any animal in the nerve-to-nerve suture, nerve graft or muscle graft groups.

An alternative explanation for the failure to record tension or electrical activity in the soleus muscles of the rats in the aforementioned groups is that 150 days was insufficient time for reflex activity to have returned after nerve regeneration. It is known that maximum recovery of muscle takes longer after nerve injuries of the neurotmesis type than after axonotmesis (Myles, 1990; Gutmann *et al.*, 1942); however it is unlikely that the recovery time allowed in the experiments presented here was not long enough for the return of the tonic vibration reflex in the nerve-to-nerve suture, nerve graft or muscle graft groups. Good recovery of isotonic muscle twitch contraction was obtained by 150 days after nerve-to-nerve suture, nerve graft and muscle graft, in both non-diabetic and diabetic animals (see chapter 6). Also, good recovery of the peroneal receptive field was found 150 days after nerve-to-nerve suture, nerve graft and muscle graft in non-diabetic rats, and after nerve-to-nerve suture in diabetic rats (chapter 5). Barker, Scott and

Stacey (1986) said that the delay between injury and recovery in human limbs averages 13 weeks after nerve crush and 16 weeks after transection and suture. Obviously the recovery time will depend on the point of injury (with the recovery time being greater for more proximal injuries); however, the time allowed in the experiments presented here was perfectly long enough to allow regeneration and recovery of both afferent and efferent nerve fibres. Banks *et al.* (1985) found that the reinnervation of muscle spindles, after nerve transection and suture of the common peroneal nerve in cats, was complete by the twelfth week.

It could be argued that the reason for the failure to record reflex tension in the soleus muscles of the animals used the experiments presented here was that the animals were not decerebrate and, consequently, anaesthetic had to be used in the experiments. The reflex responses would not have been heightened because the animals were not decerebrate (see chapter 7.1.5) and anaesthetic may have suppressed any reflex responses to the extent that they remained undetected. It is thought that this explanation for not elliciting a reflex tension in the soleus muscle of the nerve-to-nerve suture, nerve graft or muscle grafted animals is unlikely. The anaesthetic used in the experiments presented in this thesis was a mixture of Hypnorm and Hypnovel (see chapter 2.3). Hypnorm contains fentanyl citrate which acts by producing an inhibitory effect on nervous transmission from nociceptive afferents (Rang & Dale, 1987) and, therefore, it is very unlikely that this drug would have affected the tonic vibration reflex, although the response to nociceptive stimuli would not have elicited a reflex reaction (see chapter 2.3). Hypnovel contains midazolam hydrochloride which is a benzodiazepine. Binding sites for benzodiazepines exist mainly in the cerebral cortex, limbic system and mid-brain (Rang & Dale, 1987). Benzodiazepines can cause a decrease in muscle tone and it is likely that

the effect of such drugs on the tonic vibration reflex would be to cause a decrease in muscle tone, possibly by decreasing fusimotor excitation and, therefore, causing a decrease in the responsiveness of the primary endings of Ia afferents to vibration. However, the spinal monosynaptic reflex arc would be unaffected and Bianconi and Van der Meulen (1963) were able to excite primary endings in the muscles of their anaesthetized cats by vibration. Nevertheless, it is most likely that the tensions that were recorded in the experiments presented in this chapter were smaller than if the animals had been decerebrate; this is discussed below.

Since the first publication on the subject of lack of proprioceptive function after recovery from nerve transection injury and repair (Carrick, Fullarton and Glasby, 1992), there have been other reports of similar findings. Cope, Bonasera and Nichols (1994) found that the stretch reflex could not be induced by ramp-hold-release stimuli in the soleus or lateral gastrocnemius muscles, 3 years after nerve-to-nerve suture of the nerve common to both of these muscles, and these authors had successfully performed intercollicular decerebration on their cats. Cope and Clark (1993) found that the stretch reflex did return after nerve crush injuries. In experiments similar to the experiments presented here, Barker, Banks and Berry (1993) used vibratory stimuli applied to the peroneus brevis of cats to elicit a reflex response in the same muscle. They investigated the results approximately 26 weeks after repairing the common peroneal nerve with various types of graft and, in concordance with the findings of Cope *et al.* (1994) and the work presented here, Barker *et al.* (1993) also failed to evoke a reflex response in the muscles of their animals after nerve transection and repair.

From the results of the experiments presented here, if the mean tension evoked by vibration of the soleus muscle is expressed as a

percentage of the mean tetanic tension produced by the same muscles, the percentages obtained from each group are not dissimilar. The mean reflex tension expressed as a percentage of the mean tetanic tension from the non-diabetic control group was 17.63% and 20.87% for the non-diabetic crush group; the equivalent percentages for the diabetic control and crush groups were 15.99% and 16.74%, respectively. Barker *et al.* (1993) found the reflex tensions produced in the peroneus brevis muscles of their control animals were 27% and 40% of the tetanic tensions produced in the same muscles. Although these values were larger than those reported in the experiments presented here and there were obvious differences in the muscle type and species, the cats of Barker *et al.* (1993) had also been decerebrated. It can be seen from table 7.1 that the mean reflex tensions produced by the soleus muscles of the diabetic animals were somewhat smaller than the reflex tensions produced by the non-diabetic animals. When the mean reflex tensions are expressed as percentages of the mean tetanic tensions, the values obtained for the diabetic animals were a little smaller but not unlike those obtained for the non-diabetic animals. Although it would be a mistake to infer too much from the results (considering that the animals were not decerebrate), it is likely that the recovery of reflex function and the subsequent tension produced in the soleus muscle was not significantly poorer in diabetic animals than in non-diabetic animals.

The reasons why the stretch reflex should be undetectable after nerve transection injury and repair is a matter for conjecture. Eccles, Eccles and Shealy (1962) demonstrated the existence of monosynaptic excitation of motoneurons by afferent fibres that had regenerated and reformed muscle contact after nerve transection injuries. However, the number of afferent and efferent reconnections after nerve transection injuries is likely to be very

variable. Luff *et al.* (1988) demonstrated that full recovery of muscle force after nerve injury in cats could be obtained with only 5% of motor axons intact, therefore, it is quite possible that reasonable recovery of muscle function can be obtained with a minimal number of motoneuron reconnections and indeed, the rats used in the experiments presented here did not show any abnormalities in gait. Banks *et al.* (1985) found that the recovery of functionally identifiable muscle spindle afferents was only 25% of normal after nerve-to-nerve suture of the common peroneal nerve in cats, and the reinnervation of muscle spindles with functionally identifiable spindle afferents was only 10% of normal after repair using nerve autografts. Banks and Barker (1989) suggested that the quality of reinnervation of muscle spindles achieved after nerve section was partly dependent on the size of the nerve that was injured and the degree of afferent complexity. Therefore, it seems probable that the poorer numbers of functional afferent reconnections after nerve transection injuries, as compared to crush injuries, are the result of the degree of 'cross-wiring' that occurs. The degree of 'cross-wiring' could well explain the poor return of the monosynaptic reflex after nerve transection injuries. In addition, inappropriate reconnections might tend to produce opposite reflex activity to the myotatic response. It was mentioned in chapter 7.1.4 that stimulation of the Golgi tendon organs and excitation of the Ib afferent nerve fibres causes the inverse myotatic reflex. Banks and Barker (1989) found in their cross-union experiments that Ib afferents could reinnervate spindles in the place of the original Ia and group II afferents and respond to stretch in a similar way to Ia and II afferents. In fact, they found that 21% of spindles in peroneus brevis were reinnervated by Ia afferents and 20% by Ib afferents after complete transection and regeneration of the peroneal nerve. Therefore, it seems quite possible that regenerated Ib afferents could have

reinnervated spindles in the place of Ia or II resulting in the inverse myotatic response which is opposite to the desired physiological response after reinnervation.

It is possible that the failure of muscle spindles and/or extrafusal muscle fibres to become reinnervated by efferent nerve fibres could prevent or diminish the reflex response after nerve regeneration. Takano (1976) failed to find γ reinnervation of muscle spindles after denervating the leg muscles of the cat by freezing the sciatic nerve with dry ice. However, Scott (1987) investigated the reinnervation of muscle spindles by efferent fibres. He found that 89% of sectioned α axons successfully reinnervated extrafusal muscle fibres. γ efferents were also observed which did not generate tension in extrafusal fibres and he also found no increase in the number of skeletofusimotor fibres reinnervating muscle after nerve injury and repair.

Barker *et al.* (1993) measured the extent of annulospiral afferent reinnervation of muscle spindles and the number of primary endings that were reinnervated by Ia afferents after different types of nerve graft. They found that the restoration of Ia afferents to muscle spindles averaged only 3-15% of normal. This finding, alongside the failure to detect the monosynaptic Ia reflex in the animals used in the same experiments, led them to a similar conclusion to that of Carrick *et al.* (1992), that the reason for the absence of the stretch reflex was the poor return of primary endings and the "mismatch" of afferent reconnections.

If the degree of cross-wiring is the factor which controls reformation of sufficient numbers of monosynaptic reflex arcs to allow the recovery of a recordable stretch reflex after nerve transection injuries, there would be a greater probability of the return of a stretch reflex after nerve transection of a small nerve or a nerve containing only afferent or efferent fibres. It is

known that, by stretching either the lateral gastrocnemius, the medial gastrocnemius or the soleus muscle, a heterogenetic contraction is induced in the other two muscles. Importantly, Cope *et al.* (1994) found that after transection and repair of the nerve serving both the soleus and lateral gastrocnemius muscles, stretching of the medial gastrocnemius muscle, whose nerve supply was intact, had an excitatory effect on the reinnervated lateral gastrocnemius and soleus muscles although the responses were smaller than normal. However, stretching the soleus muscle did not produce heterogenetic excitation of the lateral gastrocnemius muscle and only minimal excitation of the medial gastrocnemius. Cope *et al.* (1994) suggested that their findings may be due to incomplete recovery of spindle afferents which results in a stretch signal which is "too weak to bring motoneurons to threshold".

In conclusion, it was found that the tonic vibration reflex was recovered after nerve crush injuries but not after nerve-to nerve suture, nerve graft or muscle graft injuries. The failure of return of the tonic vibration reflex after nerve transection and repair is most likely to be a combination of a reduced number of functional reconnections being made after reinnervation of the muscle investigated and also 'cross wiring' of regenerating axons such that inappropriate nerve reconnections are made.

CHAPTER 8

Assessment of Nerve Blood Flow

8.1 Introduction.

8.1.1 Aims.

It is possible that peripheral nerve lesions in diabetes mellitus have a vascular aetiology and that peripheral neuropathy seen in patients with diabetes is, in fact, ischaemic neuropathy. The metabolic hypothesis gained popularity amongst researchers particularly with the finding that aldose reductase inhibitors prevented the decrease in nerve conduction velocity found in diabetic peripheral nerve (Suzuki, Nomura, Hirata, Toyota, Goto, 1990) but in recent years the vascular hypothesis has regained favour. In parallel with the vascular hypothesis of neuropathic change in diabetic nerve, is the possibility that tissue ischaemia could contribute to the poorer rates of regeneration which have been reported in experimental models of diabetes (Longo *et al.*, 1986; Ekström & Tomlinson, 1989) and also to the poorer recovery of function, reported in this thesis, after nerve injury and repair in STZ diabetic rats. Tissue ischaemia can arise in a number of ways, including from reduced blood flow, which has been reported in STZ diabetic rats (Tuck, Schmelzer and Low, 1984; Maxfield, Cameron, Cotter and Dines, 1993). It was the aim of the work presented in this chapter to measure blood flow through the left sciatic nerve in STZ diabetic rats, 150 days after applying different methods of nerve injury and repair, and compare the results with similarly operated non-diabetic animals.

8.1.2 Blood Supply of Peripheral Nerve.

The blood supply of peripheral nerve depends a great deal on the blood vessels which are anatomically juxtaposed to the nerve. Hence, blood vessels destined to supply peripheral nerves often arise as branches from nearby main arterial trunks and from arteries which cross the nerve; and less frequently, from muscular branches of arteries (Adams, 1942). For this reason, the blood supply of nerve tends to be regional and although anastomosis does occur between adjacent nutrient arteries, the physiological significance of such vascular communication is debateable (Adams, 1942). The vessels arising from arteries which run along the length of a nerve but external to it form the extrinsic blood supply. Nutrient arteries divide either on or within the epineurium into ascending and descending rami. These vessels pass in opposite directions, often taking a very tortuous course and branch to give rise to the arterioles. These vessels form an arteriolar meshwork within the perineurium, from which the capillary plexuses are derived. According to Adams, only the capillary plexuses enter the endoneurium, however, Rundquist, Smith, Michel, Ask, Oberg and Rapoport (1985) identified vessels with diameters over 25 μm, in the endoneurium of rat sciatic nerve, which were unlikely to be capillaries. The anastomosing network of vessels in the epineurium and perineurium which arises from the extrinsic circulation, and the network of capillaries in the endoneurium together form the intrinsic circulation or *vasa nervorum* (Beggs, Johnson, Olafsen and Watkins, 1992). The belts of tight junctions of the perineurium, and the tight junctions which connect the endoneurial cells of capillaries, serve to form the blood-nerve barrier (Wadhwani & Rapoport, 1990).

8.1.3 The Role of Decreased Nerve Blood Flow and Endoneurial Hypoxia in Diabetic Neuropathy.

Okada (1905; see Adams, 1942) found that ligation of the inferior gluteal artery in rabbits resulted in degeneration of the fibres of the sciatic nerve; however, Adams (1943) found no histological changes on examination of the sciatic nerve of his rabbits between 6 days and 1 month after ligation of the inferior gluteal artery. In further experiments, Adams (1943) ligated further sources of blood supply to the rabbit sciatic nerve and found that there were gross abnormalities in only 2 out of 12 nerves; and 3 showed a small number of degenerated fibres. He claimed that it is not likely that occlusion of one blood vessel of the extrinsic circulation would produce ischaemic changes in tissue of peripheral nerve because of the rich anastomotic network of the intrinsic circulation. Myers, Heckman, Galbraith and Powell (1991) found evidence of demyelination in 32 of the 55 rat nerves they studied after inducing ischaemic conditions by stripping the *vasa nervorum* from the outside of the nerve. In contrast to events in the central nervous system: when hypoxia resulted in decreased vascular resistance, Wadhwani and Rapoport (1990) stated that the effects of hypoxia on peripheral nerve blood flow are not as clear. They suggested that this could be because the demand for oxygen in peripheral nerve is less than in central nerves, and in hypoxic conditions the blood supply of oxygen is still enough for the metabolic requirements of peripheral nerve. The work of Low and Tuck (1984) on the blood flow of the sciatic nerve in rats demonstrated, by the hydrogen clearance technique, that the blood flow of peripheral nerve was high in relation to its metabolic activity and they claimed that this was even more apparent if the blood flow of the peripheral nervous system is compared to the blood flow of the central nervous system. These authors

found that the oxygen requirements of the central nervous system are 20 times that of the peripheral nervous system and yet, the blood flow is only 6 times that of peripheral nerves. From this evidence it seems unlikely that a decrease in blood flow of peripheral nerve could cause sufficient hypoxia to bring about degenerative changes, unless the decrease were catastrophic.

In contrast, some authors have reported that the changes seen in diabetic nerve, including segmental demyelination (Seniviratne & Peiris, 1970) could be explained by tissue hypoxia. Tuck *et al*. (1984) argued that most, if not all, of the biochemical changes seen in experimental diabetic neuropathy could be explained if the oxygen supply to nerve was impaired. These authors demonstrated a decrease in the blood flow of the sciatic nerve in diabetic rats and a decrease in endoneurial oxygen tension, 4 months after administration of STZ. Seniviratne (1972) showed that in the alloxan diabetic rat there was impairment of the perineurial barrier and an increase in the permeability of the endoneurial vessels which was demonstrated by the passage of labelled albumen into the endoneurium. He argued that an increase in the permeability of the blood-nerve barrier could lead to extravasation of protein and subsequent osmotic effects which would cause an increase in the endoneurial fluid pressure. This increase in fluid pressure would, in turn, inhibit capillary filtration and formation of endoneurial tissue fluid, thus resulting in tissue hypoxia and cell damage. Also, nerve oedema as demonstrated by Jakobsen (1978) in his STZ diabetic rats could cause an increase in intercapillary distance and increase endoneurial hypoxia. However, Seneviratne (1972) found that the segments of leaking capillary or perineurium were interspersed with lengths where the permeability seemed unimpaired and Kihara, Schmelzer, Poduslo, Curran, Nickander and Low (1991) found no impairment of the blood-nerve barrier

function for albumin in their STZ diabetic rats. Although thickening of capillary basement membranes has been reported to be increased in humans as a function of the duration of diabetes (Sima, Yagihashi and Greene, 1990) and although this could decrease the rate of perfusion of oxygen into the endoneurium, the same changes have not been reported in STZ diabetic rats.

Low, Schmelzer, Ward and Yao (1986) found that in rats subjected to chronic hypoxia there was both a decrease in conduction velocity and development of resistance to ischaemic conduction block. They argued that because the nerve myoinositol and sorbitol levels were normal, the physiological changes must have been due to chronic hypoxia and that this hypoxia could have been the cause of the changes seen in the diabetic nerve. Low *et al.* (1986) said that in their experiments, where rats were exposed to 10% oxygen in an airtight chamber, the measured endoneurial oxygen values from the rats were 40-50% of normal values and the reduction in conduction velocity was approximately 10% (from 47.1 to 42.2 $m\ s^{-1}$). However, Tuck *et al.* (1984) found that, in their STZ diabetic rats, the endoneurial oxygen tension was reduced by only 25.8% and the reduction in nerve conduction velocity was 16.26%. Both of these experiments were performed in the same labaratories and differences in results were unlikely to have been due to differences in methods or labaratory conditions; therefore, the differences in the results obtained from experimental diabetic rats, as compared to those obtained from hypoxic rats, tended to suggest that there must have been some other factor in operation besides a decrease in endoneurial oxygen tension which had caused the physiological changes in peripheral nerve.

8.1.4 Changes in the Vasa Nervorum and their Innervation in Streptozotocin Diabetes.

The *vasa nervorum* of the extrinsic circulation have been shown to be affected by adrenergic stimulation (Hotta, Nishijo, Sato, Sato and Tanzawa, 1991), topical application of adrenaline (Myers & Heckman, 1989) and topical application of local anaesthetics (Kalichman & Lalonde, 1991). Importantly, there have been reports of altered responses of the vasculature, in STZ diabetes, to different substances including noradrenaline (Tomlinson, Gardiner, Hebden and Bennett, 1992) and an increased α_2 adrenoceptor-mediated vascular contraction (Scarborough, 1983). Milner, Appenzeller, Qualls, Burnstock (1992) found that the neuropeptides in the perivascular nerves of *vasa nervorum* supplying various nerves in STZ diabetic rats were different in their vulnerability. The sciatic nerve of STZ diabetic rats showed a decrease in the density of neuropeptide Y and increase in substance P and calcitonin gene-related peptide levels. These authors suggested that the change in the autonomic control of blood vessels serving peripheral nerve in diabetics may contribute to the pathogenesis of the disease. The transperineurial and epineurial vessels of peripheral nerve are innervated by plexuses of unmyelinated axons and Beggs *et al*. (1992) observed denervation of these perivascular structures serving the *vasa nervorum* in human diabetics. They proposed that denervation of the unmyelinated nerve plexuses could result in a lack of neurogenic control over the *vasa nervorum* and consequently, could lead to endoneurial hypoxia or ischaemia. This hypothesis, involving the loss of perivascular axons which leads to abnormal vasoconstriction of the *vasa nervorum* and subsequent endoneurial hypoxia and neuropathy, is quite interesting if the reasons for the initial damage to the unmyelinated axons are considered. Why should the unmyelinated axons be vulnerable to

damage in diabetes? Could the aetiology have a vascular or metabolic basis? Cameron, Cotter and Robertson (1989) found that chronic low frequency stimulation of nerve, in rats that had received injections of STZ 3 months previously, caused an improvement in the reduced conduction velocity. One reason for this increase could be attributed to vasodilatation of the *vasa nervorum* which leads to a decrease in endoneurial hypoxia and a susequent improvement in the reduced nerve conduction velocity. Cameron, Cotter, Ferguson, Robertson and Radcliffe (1991a) investigated the effects of an α-adrenergic receptor blocker on nerve conduction velocity and vascular supply in STZ diabetic rats. They found that treatment with prazosin from the time of induction of diabetes limited the decline in conduction velocity to only 5% but prazosin treatment given 1 month after the induction of diabetes did not alter the reduced sensory conduction velocity, although the motor conduction was improved. They also demonstrated that prazosin treatment caused a 20% increase in endoneurial capillary density in STZ diabetic rats but not in non-diabetic animals. Other authors have found an increase in capillary density and blood flow in STZ diabetic rats, which correlated with varying degrees of recovery of the reduced conduction velocity, using different treatments: an angiotensin II receptor blocker (ZD 8731) (Maxfield *et al.*, 1993); ω-6 essential fatty acid (Dines, Cotter and Cameron, 1993); prostacyclin analogue iloprost (Cotter, Dines and Cameron, 1993); aminoguanidine (Kihara *et al.*, 1991) and evening primrose oil (Efamol) (Cameron, Cotter and Robertson, 1991b). It should be noted that, apparently, STZ diabetes does not cause a decrease in the capillary density of nerve as compared to non-diabetic animals. Furthermore, the increase in capillary density observed using various treatments in STZ diabetic rats was over and above that seen in both non-diabetic and STZ diabetic animals: 20% with prazosin

(Cameron *et al.*, 1991a), 11% with ω-6 essential fatty acid (Dines *et al.*, 1993), 22% with evening primrose oil (Cameron *et al.*, 1991b), 15% with angiotensin II blocker (ZD 8731) (Maxfield *et al.*, 1993), 28% with the prostacyclin analogue iloprost (Cotter *et al.*, 1993). Therefore, it would seem that the tissue hypoxia, suggested as being the cause of neuropathic change, in the nerves of STZ diabetic rats is caused by something other than a decrease in capillary density, although it appears that a decrease in endoneurial oxygen tension does contribute to the physiological and biochemical changes seen in experimental diabetes.

8.1.5 Effects of Decreased Vascularity and/or Tissue Hypoxia on Nerve Regeneration.

Tuck *et al.* (1984) argued that the cause of diabetic neuropathy could have its basis in endoneurial hypoxia which produces a deficit in neuronal energy compounds. Consequently, a deficit in cell ATP, NADH *etc.* could affect the energy-requiring activities of the cell, including axonal transport. Low *et al.* (1986) proposed that the most likely cause of a decrease in nerve conduction velocity in diabetes was a decrease in axonal diameter and that this could be explained by a decrease in axonal transport. The results of the experiments presented in chapter 4 make this hypothesis seem unlikely, because the small decrease in axon diameter, found in the STZ diabetic rats used in the work presented here, was more likely to be due to tissue shrinkage than axonal dwindling. The decrease in axonal transport due to tissue hypoxia could explain the decrease in nerve regeneration observed in STZ diabetic animals (Longo *et al.*, 1986; Ekström *et al.*, 1989) and possibly the poorer recovery of sensory and muscular function seen in peripheral nerve after grafting (see chapters 4 and 5).

The importance of the blood supply in nerve regeneration does not appear to have been extensively investigated. The difference in recovery of function after repairing peripheral nerve with vascularized nerve grafts, as compared to non-vascularized (free) nerve grafts, might indicate more about the benefits of having a good blood supply to peripheral nerve during regeneration. However, the results from experiments on animals have been varied. Shibata, Tsai, Firrell and Breidenbach (1988) found that the recovery of motor function and numbers of axons in rabbit median nerve were greater after using vascularized nerve grafts than non-vascularized grafts. However, Seckel, Ryan, Simons, Gagne and Watkins (1986) found no difference in the anatomical indices of recovery after using vascularized nerve grafts. Hems (1993) found a small but not significant improvement in the recovery of cutaneous receptive field area and peak twitch tension after using vascularized nerve grafts in rabbits as compared to free nerve grafts. If vascularized nerve grafts prove to be more successful than non-vascularized grafts, the potential use of such grafts for repairing larger nerve gaps and more proximal injuries is enormous. In the experiments presented in this thesis, the blood flow was measured in peripheral nerve, at the site of nerve injury, 150 days after repair operations had been performed, so that a comparison of the recovery of neural blood flow after different types of nerve injury and repair (see chapter 2.5) could be made.

8.2. Materials and Methods.

8.2.1 Principles of Doppler Flowmetry.

Blood flow was recorded using the Moor blood flow monitor model MBF3D/42 (Moor Instruments Ltd, Axminster, U.K.). The meter works on the Doppler principle that electromagnetic light waves scattered from moving objects will experience an apparent shift in frequency and this frequency is related to the velocity of the moving object. In this case the electromagnetic waves are produced from a 3 mV semi-conductor laser at wavelengths of red or near infrared light (780-810 nm). The light is directed by an optical fibre to the tissue where it is scattered by moving blood cells. The scattered light is collected by one or more optical fibres within the probe; these fibres are arranged in parallel with the emitting light fibre.

The shifted frequencies are mixed with unchanged frequencies reflected from stationary objects. Such mixing results in interference patterns and in beating between frequencies.

When two frequencies are mixed there are points at which the waveforms are exactly in phase. By the principle of superposition the resultant amplitude is at a maximum. Similarly, when the two waveforms are exactly out of phase, the resultant amplitude is at a minimum. The variation between these maxima and minima is the effect called beats.

If the period of the beats is T, the beat frequency is $1/T$ and is calculated from the equation:

$$1/T = f = f_1 - f_2$$

where: f_1 = the unchanged frequency

f_2 = the shifted frequency

f = the beat frequency

If a large number of cells is travelling at a particular velocity, there will be more of the associated frequency of light present. The beats produced will be of greater amplitude than if only a few cells are travelling at that velocity.

The collected light strikes a photodetector plate and a photodetector current is produced. This current has both AC and DC components, both of which are analysed to provide information about blood flow. It is the frequencies present in the AC component which indicate the different velocities of particles, and the amplitudes of these frequencies which indicate the number of particles. The product of the number of moving red cells and their mean velocity gives a value called flux. The flux value is indicative of flow but does not equal it. This is because the flux measurement is based on the number of red cells passing beneath the probe and not the total volume of fluid; the flux measurement will, therefore, be affected by the haematocrit when flow is not. The problem of the units of flux are discussed below.

This flux value is calculated from the information recorded over a set time period called the time constant. If the time constant is too small, the pulsatile nature of the blood flow will be displayed on the recorded trace of

flux. By increasing the time constant, the signal is averaged over a longer time and thereby eliminates the pulsatile changes of flux and a more constant trace is produced.

Problems associated with Doppler Recording.

(a) Units. The principal disadvantage of the Doppler method of recording blood flow is that it is impossible to calibrate the recorded measurements of flux in absolute units. One reason for this is that sensitivity to rate of flow varies depending on the distance of the vessel from the tip of the recording probe. It is for this reason that Moor Instruments (MBF3D User Manual) suggest that "it is best not to use the absolute flux values but to measure relative to a baseline, or monitor the dynamic response to a test". In our particular experiments the percentage change in flow after application of a vasoconstrictor drug was recorded.

(b) Insensitivity to direction of flow. Blood cells passing at the same velocity as each other but in opposite directions will cause positive and negative Doppler shifts, both of which will cause a beating of the same frequency. Therefore, the system is insensitive to direction of movement. This is important if the recording is obtained from information received from two vessels with flow in opposite directions *i.e.* this will be high in a capillary bed but low where a large vessel is present.

(c) Susceptibility to concentration changes. In some cases photons will collide with more than one blood cell causing more than one Doppler shift. This occurrence will produce artefactual frequencies. The probability of multiple collisions happening increases with an increase in blood cell concentration. This could happen if the experimental animal loses a

significant amount of fluid through evaporation from its open wound. It is for this reason that prior to each experiment, 10 ml of 0.9% saline was injected subcutaneously at the scruff of the animal's neck to replace, by slow absorption, any fluid lost from the operation site.

Changes in blood cell concentration will also affect the DC level (see (d) below).

(d) Variation in intensity of incident light. If the intensity of the incident light is large, the beats produced will be larger than if the intensity is smaller. This is due to the principle of superposition explained earlier. The power of the beat frequency varies as the square of the incident light intensity. The DC component of the photodetector current is used as an indication of the incident light intensity and the changes in the AC component about this DC level is used to give information about blood flow. However, changes in concentration of blood cells will affect the DC level. This is because an increase in concentration will cause an increase in the amount of incident light absorbed and a concommitant decrease in the intensity of light reaching the photodetector; hence, the DC level will be artefactually lowered.

(e) Noise. When there is no light hitting the photodetector plate, there will still be background noise due to thermal effects of free electrons moving around the plate. This dark noise will contribute to the DC component of the signal. This type of noise can be compensated for by subtracting an offset from the DC level. Another form of noise called shot noise contributes to the AC component of the photodetector current. Simultaneous bombardment of the photodetector plate by more than one photon, or thermal movement of electrons from the plate colliding at the same time as a photon, will

produce anomalies in the photodetector current. This type of noise increases as the intensity of light increases as there is more probability of such coincidental collisions occuring. There is no compensation for the AC noise component.

Efforts were made to keep the room temperature constant in each of the experiments, thereby keeping difference in thermal effects, from one experiment to the next, to a minimum.

(f) Interference from external light sources. Mains operated light sources can produce fluctuations in light intensity which may interfere with the collected light, however, normal room lighting is not considered a problem in causing interference (Vongsavan & Matthews, 1993). No other sources of light except normal room lighting was used in this work.

(g) Movement artefact. If the tissue or the probe is moved during recording, there will be extra Doppler shifts produced from light reflected off of the surrounding tissue. The extra frequencies produced will cause anomalies in flux calculations.

In this work, movement artefact was kept to a minimum by holding the probes rigidly in micromanipulators (see below) and taping the animals' legs to the operating table.

8.2.2 Preparation of Experimental Animals.

Each rat was anaesthetized and both sciatic nerves were exposed (chapter 2).

With the aid of an operating microscope (Weck Fibermatic 0902A1, Long Island City, New York) and microsurgical instruments the sciatic nerve

was freed from the muscle bed. Care was taken not to disrupt blood vessels surrounding the nerve, however, it was inevitable that some vessels entering the nerve from the surrounding tissue were disturbed. To prevent haemhorrage, these vessels were sealed by pressing the tips of fine forceps over the cut ends of the vessel; the use of a diathermy to stop bleeding was thought to be too damaging and disruptive.

For consistency, measurements of flux were made from the same point of each nerve. This point was 0.5 cm from where the adductor brevis crosses the sciatic nerve. A 1 cm x 0.5 cm sheet of black plastic was inserted, from this point extending distally, beneath the nerve.

The animal was laid face down, on a heated table (Harvard Apparatus Ltd, Edenbridge, U.K.). On either side of the table was a Huxley micromanipulator (Goodfellow, Cambridge Science Park, Cambridge, U.K.) with x, y and z axes of movement. The hind limbs of the rat were loosely taped to the table so that the sciatic nerve on each side lay parallel to the y axis of movement of the appropriate micromanipulator. Two Doppler probes, each with an external diameter of 1.5 mm and optical fibre diameter of 0.2 mm, were connected to the Moor flow meter MBF3D/42 (Moor Instruments, Axminster, U.K.) and held in place by the micromanipulators. With these it was possible to position the probes, lightly and accurately, over the sciatic nerve at the point where the black plastic sheet had been inserted. Using the y axis of movement the probe could be moved 1 mm at a time along the nerve.

8.2.3 Recording of Sciatic Nerve Blood Flux.

The Moor flow meter MBF3D/42 (MBF3D) was set up with a time constant of 0.5 s. The flow meter can be set to display data at different rates; at the slowest display rate of 1 data point being recorded every 4 s,

recordings can be stored for 21 hours. In this work, the display rate was set at 5 Hz. Two pages of the monitor screen i.e. 96 s of trace were recorded, after which time the probes were moved 1 mm in a distal direction along the nerve. This process of recording-moving was repeated up to ten times. The traces were printed out and also stored on to disc using the Moorsoft MBF3D Data Handling Software package (Moor Instruments Ltd, Axminster, U.K.). Inevitably the movement of the probes caused a simultaneous movement artefact but the recording always returned to a smooth trace. The smooth part of the trace was averaged for each mm along the nerve.

The nerve on both sides was flooded with 0.2 ml of methoxamine hydrochloride (Vasoxine; The Wellcome Foundation Ltd, London, U.K.). Methoxamine hydrochloride is a non-selective α-agonist. This drug was chosen because of its effect on both pre-synaptic α_2 and post-synaptic α_1-receptors. Stimulation of α-adrenoceptors causes constriction of all smooth muscle (with the exception of that of the gastro-intestinal tract) by causing an increase in free intracellular calcium concentration. The α-receptors involved are mainly of the α_1 variety, however vascular smooth muscle possesses both α_1 and α_2 types. Therefore, addition of methoxamine to nerve will cause vasoconstriction of the *vasa nervorum* and cause a decrease in blood flow. As stated earlier, "It is best to monitor the dynamic response to a test" when using the Doppler flow meter to record blood flow. Hence, the reduction in flux measurement for each nerve was recorded.

After application of the drug the flux levels monitored by the MBF3D fell almost immediately and reached a minimum within a few minutes. When the flux levels settled at their new level, a swab was used to remove excess fluid from around the nerve. The probes were returned to the same position as at the beginning of the previous measurements. Flux levels were

recorded and the probes were moved sequentially 1 mm at a time along the nerve as has been described above. Up to ten individual recordings were made and each trace was averaged, ignoring the disturbances due to movement artefact. Figure 8.1 is a trace of the blood flux recording from a rat sciatic nerve before and after the administration of methoxamine.

8.2.4 Monitoring of Blood Pressure.

Passage of methoxamine into the general circulation will cause constriction of large arteries and veins, producing an increase in peripheral resistance and resulting in an increased systolic and diastolic arterial pressure. Baroreceptor reflexes occuring in response to blood pressure changes will then result in a bradycardia. Any change in cardiac output is likely to affect blood flow through the *vasa nervorum*. Therefore, it was considered appropriate to see if methoxamine applied locally to the nerve had a generalized effect on blood pressure. Carotid artery blood pressure was monitored using a NI108T1 isolated pressure transducer (see below). The duration of action of methoxamine was also observed by recording the length of time before blood flow began to increase again.

Exposure of the carotid artery.

The rat was prepared and anaesthetized (see chapter 2). The neck area was shaved using electrically operated clippers (Oster Professional Products, Milwaukee, U.S.A.). A vertical incision was made extending from 0.5 cm below the mouth to the manubrium sterni. The skin was retracted to expose the thymus gland which extends in to the neck in the rat. This structure was freed from its distal attachments and held out of the way of the operation site using artery forceps. A vertical incision was made through the sternomastoid muscle and retraction of this muscle exposed the

trachea. The left carotid artery is situated lateral to the trachea in the carotid sheath.

Simultaneous Recording of Carotid Artery Pressure and Sciatic Nerve Blood Flow.

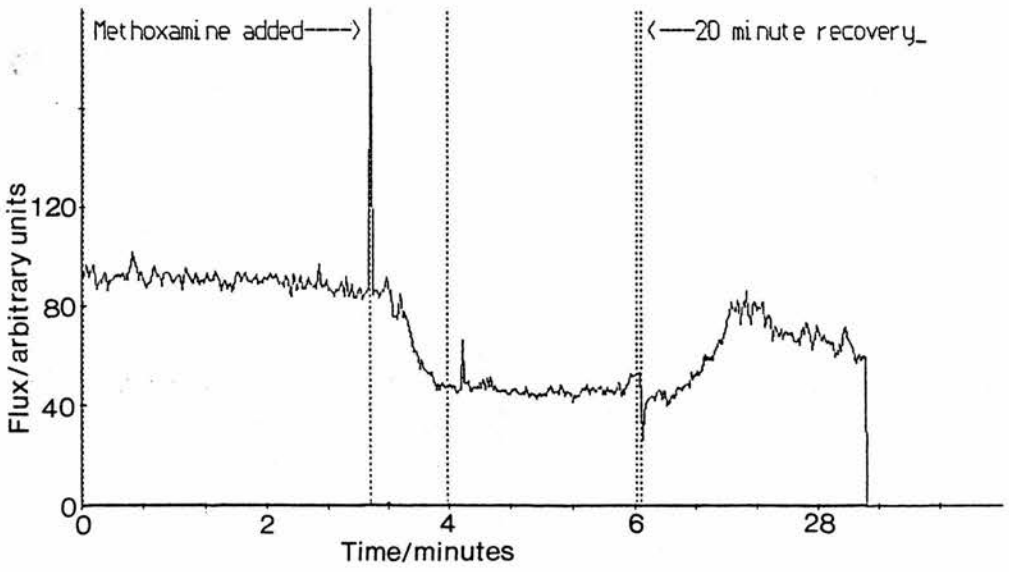
After freeing the left carotid artery from the surrounding tissue, an aneurysm needle was used to pass two lengths of 4/0 Mersilk (Ethicon Ltd, Suture Division, Edinburgh, U.K.) around the artery. These two lengths of Mersilk were subsequently used to ligate the vessel. A small transverse incision was made across the carotid artery between the two ligatures and a cannula was then introduced caudally into the artery through this incision, while simultaneously releasing and then retightening the caudal ligature to secure the cannula within the vessel. The cannula was connected to a Neurolog NL108T1 isolated pressure transducer (Digitimer Ltd, Welwyn Garden City, U.K.) by way of two gated Y-pieces arranged in series. This arrangement allowed the system to be flushed with heparinized saline to purge it of air while protecting both the cannula and the transducer.

The isolated pressure transducer was connected to a Neurolog NL108 pressure amplifier (Digitimer Ltd, Welwyn Garden City, U.K.). The output of the amplifier was fed to one of the y-inputs of a Gould 4070 digital storage oscilloscope (Gould Electronics Ltd, Ilford, U.K.).

The pressure was recorded throughout the duration of the experiment (see below).

The sciatic nerves were prepared for recording blood flow as previously described. Methoxamine was applied in the same manner as before. Flux was recorded before and after the addition of methoxamine until the vasoconstriction induced by the drug had ceased.

Figure 8.1 A trace of the recording of blood flux through the sciatic nerve of a rat before and after the administration of methoxamine.



8.3 Results.

8.3.1 Effects of Methoxamine on Blood Pressure.

The strength of the laser Doppler method lies in the detection of changes in blood flow (Westerman, Widdop, Hannaford, Low, Roberts, Kent, Sideris, Yip, Hales and Stephens, 1988). For this reason, in the experiments presented here, the vasoconstrictor methoxamine hydrochloride (methoxamine) was applied locally to the sciatic nerve to cause a decrease in blood flow. One of the potential problems associated with comparing measurements of blood flow between animals is that differences in blood pressure can cause unwanted differences in blood flow that will inevitably affect results. Passage of methoxamine into the general circulation could affect blood pressure because the drug is known to cause hypertension and reflex bradycardia (Rang & Dale,1987). It was, therefore, considered necessary to monitor the blood pressure of rats to see if methoxamine, applied locally to peripheral nerve, had a generalized effect on blood pressure. The blood pressure was monitored in 3 rats as described in chapter 8.2.4 and recording was continued for over an hour after the application of methoxamine to the sciatic nerves. In none of the rats did the blood pressure change considerably. Figure 8.2 and 8.3 are traces of blood pressure recorded from a rat prior to, and 1 hour after the application of methoxamine to its sciatic nerves, respectively. It was, therefore, considered that in the experiments presented here, insufficient methoxamine passed into the general circulation of the rats to cause a change in blood pressure.

8.3.2 Flow Ratio.

Although the effects of methoxamine applied locally to the sciatic nerves of the rats used in the experiments presented here did not appear to alter the general blood pressure, differences in blood pressure from one rat to another cannot be eliminated. When blood flow is being considered, the equation which most closely describes flow and its determinants is Poiseuille's equation:

$$\dot{Q} = \Delta P \pi r^4 / 8\eta l$$

where: $\dot{Q}$ = flow/ m^3 s

P = pressure/ Pa

r = radius of the vessel/ m

η = dynamic viscosity/ Pa s

l = length of the vessel/ m

Poiseille's equation can be simplified to:

$$\text{flow} = \text{pressure} / \text{vascular resistance}$$

Because it is advisable to measure a change in flow, the values of flux after addition of methoxamine ($flux_m$) can be compared as a ratio to the flux before the addition of methoxamine ($flux_o$). The values of flow ratio for each animal are displayed in appendix 19, as are the values of $flux_m$ and $flux_0$ for both left/operated and right/unoperated sides.

flow ratio = $flux_m$ / $flux_0$ and is proportional to:

$$\frac{(\text{pressure}_m \times \text{vascular resistance}_o)}{(\text{pressure}_o \times \text{vascular resistance}_m)}$$

However, it is unlikely that $pressure_m$ and $pressure_o$ are very much different (see 8.3.1 above) and therefore:

$$pressure_m / pressure_o \approx 1.$$

If similarity in blood pressure before and after addition of methoxamine is assumed, the flow ratio actually describes the ratio of vascular resistances before and after addition of methoxamine, and this ratio obtained from different animals can be compared without the problems of differences in blood pressure between animals.

8.3.3 Results from Experiments on Blood Flow.

Blood flux before administration of methoxamine.

The mean, standard deviation, standard error of the mean and coefficient of variation of the values of blood flux recorded from the left sciatic nerves of the non-diabetic animals, before the addition of methoxamine, are displayed in table 8.1 and the equivalent values for the diabetic animals are displayed in table 8.2.

Table 8.1 Values of the mean, standard deviation, standard error of the mean and coefficient of variation for blood flux of the left/operated sciatic nerve in non-diabetic rats.

RAT GROUP	n	m /arbitrary units	SD	SEM	c.v.
C	5	177.74	35.33	15.80	19.88
CR	5	105.57	26.61	11.90	25.20
NN	5	69.15	14.50	6.49	20.98
NG	5	109.98	28.25	12.6	25.68
MG	5	76.18	10.72	4.79	14.07

It can be seen that the values of mean flux were highest in the control group, as compared to the operated groups, for both non-diabetic and

diabetic animals. If comparisons are made between the non-diabetic groups of animals and the diabetic equivalents, it can be seen that the mean blood flux recorded from the left sciatic nerves of the diabetic animals was smaller for each group except in the case of nerve-to-nerve suture.

Table 8.2 Values of the mean, standard deviation, standard error of the mean and coefficient of variation for blood flux of the left/operated sciatic nerve in diabetic rats.

RAT GROUP	n	m /arbitrary units	SD	SEM	c.v.
DC	5	135.50	17.78	7.95	13.13
DCR	5	81.26	21.20	9.48	26.08
DNN	5	108.71	16.80	7.52	15.46
DNG	5	105.38	9.32	4.17	8.84
DMG	5	52.45	21.10	9.43	40.22

The mean, standard deviation, standard error of the mean and coefficient of variation of the values of blood flux recorded from the right sciatic nerves of the non-diabetic animals before the addition of methoxamine are displayed in table 8.3 and the equivalent values for the diabetic animals are displayed in table 8.4.

Table 8.3 Values of the mean, standard deviation, standard error of the mean and coefficient of variation for blood flux of the right/unoperated sciatic nerve in non-diabetic rats.

RAT GROUP	n	m /arbitrary units)	SD	SEM	c.v.
C	5	172.84	50.01	22.37	28.94
CR	5	182.17	39.55	17.69	21.71
NN	5	123.18	43.54	19.47	35.35
NG	5	165.43	51.76	23.15	31.29
MG	5	191.04	11.28	5.05	5.91

Table 8.4 Values of the mean, standard deviation, standard error of the mean and coefficient of variation for blood flux of the right/unoperated sciatic nerve in diabetic rats.

RAT GROUP	n	m /arbitrary units	SD	SEM	c.v.
DC	5	139.40	38.58	17.25	27.68
DCR	5	137.02	43.34	19.38	31.63
DNN	5	164.92	25.65	11.47	15.56
DNG	5	174.95	31.70	14.18	18.12
DMG	5	160.16	57.07	25.52	35.63

From these tables it can be seen that the mean blood flux varied by as much as 35 arbitrary flux units between groups, although the flux values were not as low as those recorded from the operated sciatic nerves. The mean blood flux measured from the left sciatic nerves of the non-diabetic nerve graft group was large: 169.98 arbitrary flux units, in comparison to the muscle graft group and nerve-to-nerve suture groups where the mean blood flux was only 76.18 and 69.15 arbitrary flux units, respectively (see table 8.1). In fact, it was only in the non-diabetic nerve graft group that the values of mean blood flux recorded from the operated sciatic nerves was greater than the mean blood flux recorded from the right/unoperated sciatic nerves. In general, therefore, the blood flux was less in the operated nerves, as compared to the unoperated sciatic nerves, and was possibly a little less in the diabetic animals.

Blood flux after the administration of methoxamine.

Table 8.5 shows the values of the mean, standard deviation, standard error of the mean and coefficient of variation of the values of blood flux recorded from the left sciatic nerves of the non-diabetic animals after the

addition of methoxamine and table 8.6 shows the equivalent values for the diabetic animals.

Table 8.5 Values of the mean, standard deviation, standard error of the mean and coefficient of variation for blood flux of the left/operated sciatic nerve after administration of methoxamine in non-diabetic rats.

RAT GROUP	n	m /arbitrary units	SD	SEM	c.v.
C	5	54.83	7.09	3.17	12.93
CR	5	64.73	22.23	9.94	34.34
NN	5	48.26	9.77	4.37	20.25
NG	5	42.97	3.86	1.73	8.99
MG	5	47.70	3.83	1.71	8.03

Table 8.6 Values of the mean, standard deviation, standard error of the mean and coefficient of variation for blood flux of the left/operated sciatic nerve after administration of methoxamine in diabetic rats.

RAT GROUP	n	m /arbitrary units	SD	SEM	c.v.
DC	5	48.64	6.95	3.11	14.29
DCR	5	47.30	16.15	7.22	34.13
DNN	5	44.50	5.82	2.60	13.08
DNG	5	43.48	10.02	4.48	23.04
DMG	5	33.04	11.38	5.09	34.44

Although the values of mean blood flux were greatest in the control and crush groups in both non-diabetic and diabetic animals, there was not much difference in the values of mean blood flux among groups of animals, unlike the large differences in mean blood flux among groups before the addition of methoxamine (see tables 8.1 and 8.2). The values of mean blood flux were slightly smaller in the diabetic animals.

Tables 8.7 and 8.8 show the values of the mean, standard deviation, standard error of the mean and coefficient of variation of mean blood flux recorded from the right sciatic nerves after the administration of methoxamine.

Table 8.7 Values of the mean, standard deviation, standard error of the mean and coefficient of variation for blood flux of the right/unoperated sciatic nerve after administration of methoxamine in non-diabetic rats.

RAT GROUP	n	m /arbitrary units	SD	SEM	c.v.
C	5	67.32	14.49	6.48	21.52
CR	5	77.04	21.79	9.74	28.28
NN	5	72.37	11.82	5.29	16.34
NG	5	58.91	17.84	7.98	30.29
MG	5	70.57	5.98	2.68	8.48

Table 8.8 Values of the mean, standard deviation, standard error of the mean and coefficient of variation for blood flux of the right/unoperated sciatic nerve after administration of methoxamine in diabetic rats.

RAT GROUP	n	m /arbitrary units	SD	SEM	c.v.
DC	5	51.88	14.41	6.44	27.77
DCR	5	67.26	12.58	5.63	18.71
DNN	5	79.97	18.85	8.43	23.57
DNG	5	68.53	13.28	5.94	19.38
DMG	5	62.90	20.59	9.21	32.73

From these tables it appears that the mean values of blood flux recorded from the right sciatic nerves were larger than the values of flux recorded from the operated left sciatic nerves of the same groups of animals. The mean blood flux in the right sciatic nerves in the diabetic animals, after the addition of methoxamine, did not appear to be any

different from the values of flux recorded from the right sciatic nerves of the non-diabetic animals, after the addition of methoxamine.

Results from the values of blood flow ratio.

The values of the mean, standard deviation, standard error of the mean and coefficient of variation of flow ratio (described above) of the left operated sciatic nerves of the non-diabetic rats are displayed in table 8.9; the equivalent values obtained from the diabetic rats are displayed in table 8.10.

Table 8.9 Values of the mean, standard deviation, standard error of the mean and coefficient of variation for the flow ratio of the left/operated sciatic nerves, in non-diabetic rats.

RAT GROUP	n	m	SD	SEM	c.v.
C	5	0.335	0.137	0.061	40.84
CR	5	0.606	0.117	0.052	19.28
NN	5	0.714	0.122	0.055	17.12
NG	5	0.419	0.117	0.052	27.81
MG	5	0.643	0.124	0.056	19.36

Table 8.10 Values of the mean, standard deviation, standard error of the mean and coefficient of variation for the flow ratio of the left/operated sciatic nerves, in diabetic rats.

RAT GROUP	n	m	SD	SEM	c.v.
DC	5	0.371	0.101	0.045	27.32
DCR	5	0.605	0.207	0.093	34.17
DNN	5	0.418	0.087	0.039	20.82
DNG	5	0.408	0.065	0.029	15.92
DMG	5	0.642	0.06	0.027	9.4

Plots of the residuals of flow ratio against the fitted values of the mean for each group, showed that each group had a similar variance. Normal and half-normal plots were constructed of the values of blood flow ratio and

straight lines were obtained which demonstrated that the data were normally distributed and provided justification for the application of parametric statistics.

Results from significance tests on the values of flow ratio.

a) F test for variance.

The value of F obtained from the F test on the data of flow ratio of the left sciatic nerves was $F = 5.52$, d.f. = 9, 49. This result was significant ($p < 0.01$) which indicated that the samples were not from the same population.

b) Student's t tests on the means of flow ratio.

Non-diabetic rats.

The values of t and the significance levels of the tests performed on the values of flow ratio obtained from the non-diabetic animals are displayed in table 8.11.

Table 8.11 Table of t values and significance levels for the values of flow ratio of the left/ operated sciatic nerves in non-diabetic animals (Student's t test)

	C	CR	NN	NG	MG
C	-	t= -3.03 NS	t=-4.20 p<0.01	t=-0.69 NS	t=-3.43 p<0.05
CR	-	-	t=-1.17 NS	t=2.34 NS	t=-0.39 NS
NN	-	-	-	t=3.51 p<0.05	t=0.77 NS
NG	-	-	-	-	t=-2.74 NS
MG	-	-	-	-	-

The flow ratio was significantly larger in the muscle graft group than the control group ($p < 0.05$) and in the nerve-to-nerve suture group as compared to the control group ($p < 0.01$). Although the mean flow ratio of the crush group was considerably larger than the mean of the control group (0.606 as compared to 0.335, for the crush and control groups, respectively), these were not significantly different at the 5% level. The mean flow ratio of the nerve graft group was marginally larger than that of the control group (0.419 as compared to 0.335, for the nerve graft and control groups, respectively) but these values were not significantly different ($p > 0.05$). It has been discussed above that the mean blood flux of left sciatic nerves of the nerve graft group, before addition of methoxamine, was unexpectedly high if compared to the values of the other operated groups (see table 8.1). It is probable that the high values of blood flux contributed to the rather low value of mean flow ratio. Results from t tests performed on the means of flow ratio between operated groups were not significant ($p > 0.05$), except between the nerve-graft and nerve-to-nerve suture groups ($p < 0.05$). The aforementioned significant result is probably due to the abnormally low value of flow ratio obtained from the nerve graft group.

In general, the values of flow ratio were larger in the operated groups as compared to the control group (see table 8.9), which demonstrates that the change in flow was smaller after addition of methoxamine in the operated groups. The reasons for a smaller change in flow in regenerated sciatic nerves is discussed in chapter 8.4.

Diabetic rats.

The values of t and the significance levels of the tests performed on the values of flow ratio obtained from the left sciatic nerves of the diabetic animals are displayed in table 8.12. As in the results from the non-diabetic

animals, the values of flow ratio were larger in the operated groups when compared to the control group (see table 8.10); however, the difference between the values of mean flow ratio was smaller than for the non-diabetic groups and this was made apparent in the statistical tests where only the mean flow ratio of the muscle graft group was significantly larger than the mean flow ratio of the control, group ($p < 0.05$). The mean flow ratio of the muscle graft group was also significantly larger than the nerve-to-nerve suture group ($p < 0.05$) but although the mean flow ratio of the muscle graft group (0.642) was larger than the mean of the crush group (0.605) and the nerve graft group (0.408), they were not significantly different from each other ($p > 0.05$).

Table 8.12 Table of t values and significance levels for the values of flow ratio of the left/ operated sciatic nerves in diabetic animals (Student's t test)

	DC	DCR	DNN	DNG	DMG
DC	-	t=-2.58 NS	t=-0.09 NS	t=-0.77 NS	t=-3.42 p<0.05
DCR	-	-	t=2.49 NS	t=1.81 NS	t=-0.84 NS
DNN	-	-	-	t=-0.68 NS	t=-3.33 p<0.05
DNG	-	-	-	-	t=-2.65 NS
DMG	-	-	-	-	-

The values of mean flow ratio were larger in all of the operated groups when compared to the control groups, however, the difference was only significant between the muscle graft and the control group ($p < 0.05$). Because the initial blood flux of the control group, before the addition of

methoxamine, was lower than the blood flux of the non-diabetic control group, the mean flow ratio of the diabetic control group was larger than the mean flow ratio of the non-diabetic control group. Consequently, the values of mean flow ratio of the diabetic operated groups were too similar to the value of mean flow ratio of the diabetic control group to show any significant difference in statistical tests.

Non-diabetic *v* diabetic rats.

Table 8.13 shows the t values and significance levels of tests performed on the corresponding pairs of group means of flow ratio obtained from the left sciatic nerves of rats, from diabetic and non-diabetic populations. There was no significant difference in the change in blood flow between diabetic and non-diabetic rats from control, crush, nerve graft or muscle graft groups ($p > 0.05$). Because of adjusting the t tests for multiple comparisons, the flow ratio obtained from the non-diabetic nerve-to-nerve suture group was not significantly larger than the flow ratio of the diabetic nerve-to-nerve suture group ($p > 0.05$) although the t value approached significance ($t = 4.10$). The difference from the nerve-to-nerve suture groups is probably due to the low values of blood flux recorded from the left sciatic nerves of the non-diabetic nerve-to-nerve suture group (see table 8.1), which resulted in a high value of mean flow ratio.

Table 8.13 Table of t values and significance levels for the values of flow ratio of the left/unoperated sciatic nerves in non-diabetic animals *v* diabetic animals (Student's t test)

	C	CR	NN	NG	MG
DC	t=0.00 NS	-	-	-	-
DCR	-	t=0.45 NS	-	-	-
DNN	-	-	t=4.10 p<0.01	-	-
DNG	-	-	-	t=-0.08 NS	-
DMG	-	-	-	-	t=0.00 NS

Figure 8.2 A trace of the recorded blood pressure of a rat, prior to administration of methoxamine to its sciatic nerves

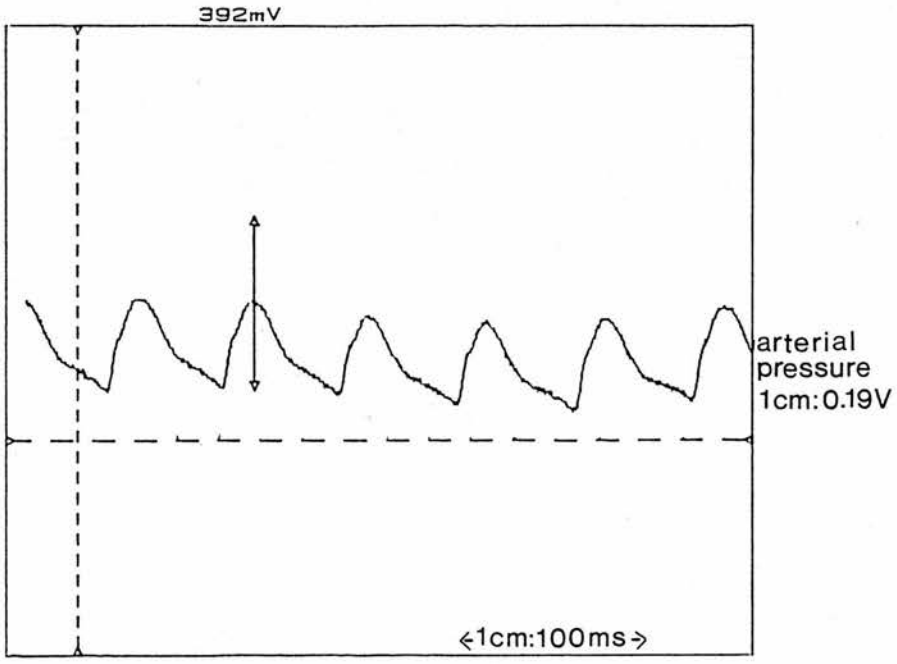
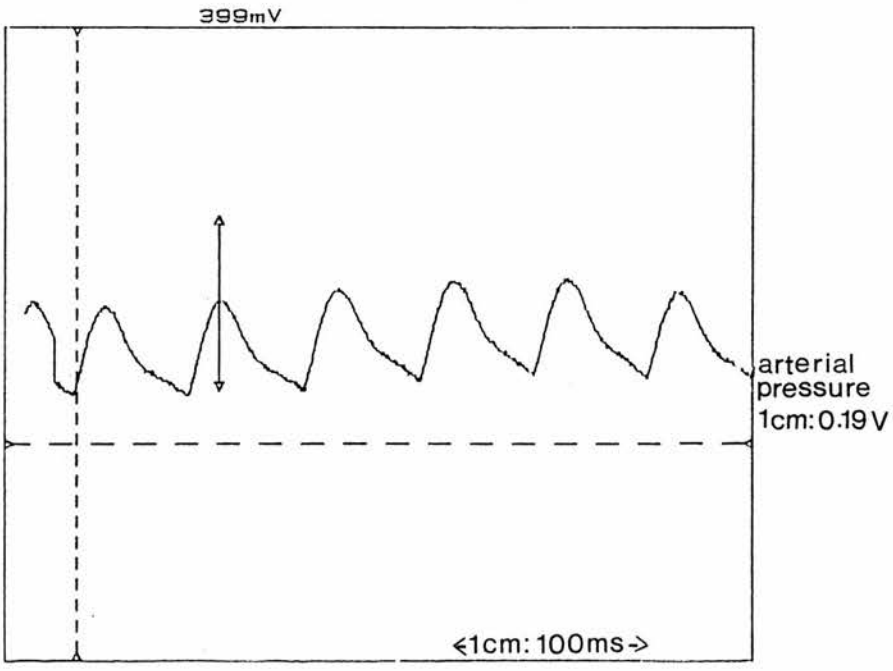


Figure 8.3 A trace of the recorded blood pressure of a rat, 1 hour after the administration of methoxamine to its sciatic nerves



8.4 Discussion.

Laser Doppler flowmetry offers particular advantages in the investigation of relative changes in blood flow in response to stimuli (Westerman *et al.*, 1988). The measurements of blood flux produced by the laser Doppler method cannot be calculated in absolute units (Vongsavan & Matthews, 1993) because the sensitivity to rate of flow depends on the distance of the blood vessels from the tip of the recording probe. Some researchers, however, have used the arbitrary units of flux to make a direct comparison between animals (Maxfield *et al.*, 1993). In the experiments presented in this thesis, the non-specific α agonist, methoxamine, was used to bring about vasoconstriction of the epineurial and perineurial blood vessels and thus, cause a change in blood flow. By this method, a relative change in flow was produced which could be compared in different groups of animals (see chapter 8.3.2). Since performing the experiments, however, it has been discovered that the vascular smooth muscle of STZ diabetes animals can show altered responses to vasoactive agents such as noradrenaline, when compared to non-diabetic animals, although the reports vary in the findings of either increased or decreased responsiveness to vasoactive agents, depending on the location of the vessels (Tomlinson *et al.*, 1992). The aim of using methoxamine in the experiments presented here was to produce a relative change in the blood flow such that a comparison could be made of blood flow in the sciatic nerve, after nerve injury and repair, between diabetic and non-diabetic populations. A change in the sensitivity of vascular smooth muscle in STZ diabetic rats to the action of α agonists would cause relative changes in blood flow in the

diabetic animals that, if compared to the relative changes in blood flow in non-diabetic rats, would not indicate a difference in blood flow after nerve injury and repair but rather an altered responsiveness of the vasculature of STZ diabetic rats to methoxamine. Therefore, it was considered advisable to discuss the differences in the recordings of blood flux between groups of animals from the non-diabetic and diabetic populations as well as discussing the relative changes in blood flow (or flow ratio; see chapter 8.3.2). It should be remembered that the values of flux are only measured in arbitrary units and that the method of recording blood flow is insensitive to direction of flow so that the values of flux, ideally, should not be used quantitatively. However, Rundquist *et al.* (1985) found from regression analysis on the recordings of blood flow in the sciatic nerves of rats using laser Doppler flowmetry and [14c] iodoantipyrine methods, that the regression line did not differ significantly from zero. They also found that by dividing the values recorded from Doppler flowmetry by the square of nerve diameter, to correct for differences in nerve cross-sectional area, the correlation of results of nerve blood flow measured by the two methods was not improved.

In the experiments presented here, the mean blood flux measured from the left sciatic nerves of the non-diabetic control group was 177.74 as compared to 135.50 obtained from the left sciatic nerves of the diabetic control animals. From these measurements it is easy to think that the blood flow of the sciatic nerve was reduced in the STZ diabetic rats and this would agree with the findings of Low *et al.* (1984) who found a reduction in the blood flow of the sciatic nerves of STZ diabetic rats using the hydrogen clearance technique and Maxfield *et al.* (1993) who found a reduction in blood flow of 47% in the sciatic nerve in STZ diabetic rats, using laser Doppler flowmetry. However, if the values of blood flux are compiled

from the right unoperated sciatic nerves of the rats used in the experiments presented here and are averaged for the diabetic and non-diabetic animals, the mean blood flux for the non-diabetic animals = 166.93 ± 9.61 (n = 25) and 155.29 ± 8.66 (n = 25) for the diabetic animals. The difference in blood flux between the two populations is, in fact, only 7%. From the work presented here, it would seem that STZ diabetes does not much alter the blood flow through the sciatic nerve and probably not enough to bring about neuropathic change. If the values of flow ratio are compiled for the right sciatic nerves and averaged, the mean flow ratio of the right sciatic nerves of all 25 non-diabetic rats = 0.444 ± 0.030 and the mean flow ratio of the 25 diabetic rats = 0.453 ± 0.035. These figures demonstrate that the change in flow after addition of methoxamine was slightly less in the diabetic animals. This result sheds doubt on the hypothesis that STZ diabetic animals may have increased responsiveness to vasoconstrictors due to loss of neurogenic control over the *vasa nervorum* which would then lead to endoneurial hypoxia (Tomlinson *et al.*, 1992) although the results agree with the findings of Scarborough and Carrier (1983) who found that contractile responses to methoxamine were unaltered by STZ treatment.

The question arises as to why Maxfield *et al.* (1993) found a reduction in nerve blood flow in the sciatic nerves of their STZ diabetic animals as compared to their non-diabetic animals. The negative aspects of using the arbitrary units measured by the laser Doppler method have already been stressed but Maxfield *et al.* (1993) also recorded values of flux from one point on each nerve only. Anomalously high values of flow could have been measured because of the possibility of the probe's being situated over a large epineurial or perineurial vessel. In the experiments presented in this thesis, the recording of blood flow was measured over

10 points so that the probability of such an anomalous recording was reduced.

Low *et al.* (1984) also recorded smaller values of blood flow through the nerves of their STZ diabetic rats. In chapter 8.3.2, the factors affecting blood flow were discussed and the possibility of blood pressure changes affecting the results of the experiments presented here was rationalized. By using the flow ratio, the effects of differences in blood pressure between groups of animals on the results are minimized and flow changes have their basis in changes in peripheral resistance. A decrease in blood flow can be caused by an increase in blood viscosity which causes an increase in peripheral resistance. Blood viscosity can be increased if haemoglobin concentration or the haematocrit are increased. Therefore, if the diabetic rats of Tuck *et al.* (1984) were dehydrated, it is possible that blood flow recordings would have been lower than control values when using the hydrogen clearance technique. In contrast, an increased haematocrit in the rats used for Doppler flowmetry measurements would probably have produced anomalously high recordings of flux because the flowmeter was calibrated for normal haematocrit. Considering the fact that the values of flux were no larger in the diabetic animals used in the experiments presented here than in the non-diabetic animals, it is probable that the animals were not significantly dehydrated. If the animals had been dehydrated, a true decrease in blood flow could have been obscured because of anomalously high recordings of flow. Increase in peripheral vascular resistance can also occur if the number of patent capillaries is reduced, however, many authors have found that the capillary density of peripheral nerve in STZ diabetic rats was no different from that in nerve of non-diabetic rats (Maxfield *et al.*, 1993; Cameron *et al.*, 1991a; Dines *et al.*, 1993; Cotter *et al.*, 1993). Low and Tuck (1984) demonstrated, using

the hydrogen clearance technique, that respiratory acidosis made no significant difference to the blood flow of the rat sciatic nerve. An increase in blood viscosity and a subsequent reduction in blood flow could also result from an increase in glucose concentration (Tuck *et al.*, 1984). Therefore, an alternative reason for the difference in results between those of Tuck *et al.* (1984) and Maxfield *et al.* (1993), and those reported in this thesis might be a difference in the severity of diabetes. During the course of the experiments of Maxfield *et al.* (1993), the mean body weight of the rats fell dramatically and the mean value of blood sugar was very high (41.8 ± 1.9 after 2 months of STZ diabetes). The body weight of the rats used in the work in this thesis gradually increased over the course of the experiments and the blood glucose levels were much lower (see chapter 3). But if parallels are to be drawn between human and experimental diabetes, surely a more comparable situation is that of poor glycaemic control rather than permanent hyperglycaemia. Also, physiological changes such as resistance to ischaemic conduction block and decreased conduction velocity occur in diabetic animals with all degrees of abnormal blood glucose and if claims are to made about the aetiology of diabetic neuropathy then the proposed mechanism should be operational in all diabetics; unless, there is more than one factor operating to cause neuropathic changes and the dominant factor is determined by the level of hyperglycaemia.

As well as demonstrating a decrease in blood flow in the sciatic nerve of their STZ diabetic rats, Tuck *et al.* (1984) demonstrated a decrease in the endoneurial oxygen tension. They suggested that the biochemical changes seen in experimental diabetic neuropathy including "reduced nerve myoinositiol concentration, decreased rate of synthesis and transport of intra-axonal proteins, reduced incorporation of glycolipids and amino

acids into myelin and excessive intracellular accumulation of glycogen" could all be explained by endoneurial hypoxia. However, Low and Tuck (1984) demonstrated that the blood flow in peripheral nerve of non-diabetic rats was high in comparison to its metabolic requirements and other evidence suggests that a reduction in blood flow would have to have been quite dramatic to cause any degenerative changes in peripheral nerves (see chapter 8.1.3). Therefore, it is to be inferred that the decrease in blood flow observed in STZ diabetic rats, as measured in the experiments presented here, is unlikely to be the dominant factor in the causation of neuropathy and could only contribute to the changes seen in the peripheral nerve of diabetic rats. The effects of a decreased blood flow may be more important in chronic human diabetes where macroangiopathic and microangiopathic changes are considerable (Tomlinson *et al.*, 1992). Rats are relatively resistant to atherosclerotic changes (Tomlinson *et al.*, 1992) and, therefore, STZ diabetic rats probably do not show the same pathological changes in the vasculature as long-term human diabetics.

The neuropathic changes in peripheral nerves that have been proposed to be caused by hypoxia of neural tissue, may not only be caused by a decrease in blood flow but also may be produced by a change in the permeability of capillary endoneurial cell membranes to oxygen. Microangiopathic changes have been demonstrated in human diabetic neuropathy (Yasuda & Dyck, 1987); changes in vascular permeability (Seneviratne, 1972) and an increase in the water content of the endoneurial space (Jakobsen, 1978) have been demonstrated in animal models of diabetes. In contrast, microangiopathic changes in STZ diabetic rats have not been reported, the number of capillaries in STZ diabetic rats has not been found to be any different from non-diabetic

controls (Maxfield *et al.*, 1993) and some authors have failed to find changes in the permeability of blood-nerve barriers in diabetic rats (Kihara *et al.*, 1991). However, numerous reports have demonstrated improvements in the decreased conduction velocity and resistance to ischaemic conduction block in STZ diabetic rats when the capillary density has been caused to increase (Cameron *et al.*, 1991; Cotter *et al.*, 1993; Dines *et al.*, 1993). Myers *et al.*, (1991) demonstrated using laser Doppler flowmetry, that removal of the *vasa nervorum* from the surface of rat peripheral nerve resulted in the reduction in nerve blood flow by 58.4% and a reduction in endoneurial oxygen tension from 22.9 ± 6.0 mm Hg to 14.2 ± 5.4 mm Hg. It is unclear how much bleeding from the nerve vasculature occurred after removal of the *vasa nervorum*. In the pilot studies of the work presented here, it was noticed that if the vessels situated externally on the epineurium were accidentally severed, there was much bleeding which resulted in, firstly, an increase in the measured blood flux, followed by a subsequent fall in measured blood flux. It was considered that the initial increase in blood flux was due to the greater velocity of blood cells moving past the probe during bleeding (the peripheral resistance is effectively decreased when a blood vessel is transected) which resulted in a greater calculation of flux. Subsequently, the volume of blood and the amount of bleeding decreased such that the recording of blood flux fell below the inital values. In their experiments, Myers *et al.* (1991) must have removed the recording probe from the nerve in order to remove the epineurium. Special care was taken in the experiments presented in this thesis not to disturb the nerve underlying the probe and to record blood flow over the same 10 points before, as after, the administration of methoxamine. This is because recordings of blood flux using the laser Doppler method are very

susceptible to movement artefact and disturbance of the nerve inevitably disrupts recording. Once the probe has been removed from the nerve it is almost impossible to replace the probe in exactly the same position as before. Consequently, differences in both the position of the probe over nerve blood vessels and the pressure of the probe on the tissue, make comparisons of blood flow before and after the removal of the probe meaningless. It was noted in the experiments presented here that even slight pressure on the nerve caused an instant decrease in the recordings, which was most likely to be because of occlusion of vessels. Therefore, it is only to be suspected that the results from the experiments of Myers *et al.* (1991) after removal of the epineurium were unreliable. However, the decrease in endoneurial oxygen tension reported by these authors is to be expected if a nerve is stripped of its nutrient vessels but such an extreme reduction in the blood supply of a nerve and the subsequent ischaemia is hardly comparable to the probable chronic hypoxia of diabetic nerve.

If the neuropathic changes seen in experimental animal models of diabetes are not due to hypoxia through reduction in blood flow alone but require, in addition, a decrease in endoneurial tissue oxygenation by some other means, the effects of the reduction in blood flow in causing the poorer rates of recovery of peripheral nerve and also in causing the poorer recovery of function after nerve injury and repair (reported in the work of this thesis) are debateable. In the work presented here, the lower values of blood flux recorded from the sciatic nerves after nerve injury and repair were not significantly poorer in the diabetic animals than in the non-diabetic animals; therefore, unless tissue oxygenation was decreased in some other way, it seems unlikely that the cause of the poorer

regeneration and recovery of diabetic nerve after injury and repair was due to poor blood supply. The recovery of function after nerve injury and repair in diabetic rats was only significantly poorer, as compared to non-diabetic rats, in the sensory system (see chapter 5) which suggests that a vascular hypothesis as the cause of poorer recovery is even less likely because it would be expected that the effects of tissue hypoxia would affect all regenerating nerve fibres.

The values of mean blood flow ratio were larger in the operated groups of animals, as compared to controls, and highest in the muscle graft groups. This finding shows that the change in blood flow of the sciatic nerve, after addition of methoxamine, was less after nerve injury and repair. Therefore, it appears that the blood supply of peripheral nerve does not return to normal after nerve injury. The smaller changes in blood flow in response to application of a vasoconstrictor could be because the newly formed blood vessels did not respond to methoxamine in the same way as the blood vessels of control nerve; possibly because the newly formed vessels were mostly endoneurial capillaries which did not respond to vasoconstrictive agents. It is thought that this was probably not the predominant reason for the smaller changes in blood flow after nerve injury because after application of methoxamine, the measurements of blood flux were reduced to approximately the same levels in all nerves, whatever type of injury had been sustained. A likely reason for the smaller changes in blood flow of the sciatic nerve in the operated groups is that the initial values of flux, before application of methoxamine, were lower in the operated groups of animals. These lower values of flux that were recorded from the sciatic nerves of animals in the operated groups, before the addition of methoxamine (see tables 8.1 and 8.2, for non-diabetic and diabetic animals, respectively) are suggestive of a much poorer blood supply after nerve injury and repair. The

poorest recovery of nerve blood flow was found in the muscle graft groups of both the diabetic and non-diabetic populations. It is possible that a poor blood supply to regenerating nerve could contribute to a poorer rate of regeneration and subsequent recovery of function. The importance of an improved blood supply on nerve regeneration and recovery of function could be studied by comparing results from experiments using vascularized and non-vascularized nerve grafts. However, as mentioned in chapter 8.1.5, the reports so far on the benefits of using vascularized nerve grafts have been conflicting. There is an alternative approach to the study of the importance of a good supply in nerve regeneration:

According to Cameron *et al.* (1991a), vasodilators have been found to increase the number of patent capillaries, increase the vessel size and finally increase angiogenesis in skeletal muscle and heart muscle. Experiments could be designed where animals are treated with vasodilators or other angiogenic stimulators e.g. prostaglandin E1 or E2 (Form & Auerbach, 1983) throughout a period of nerve regeneration. Any changes in the blood supply of peripheral nerve, and the value of increased nerve blood supply on the recovery of function after nerve injury and repair, could be assessed in a manner similar to the methods of the experiments presented here. In addition, the endoneurial capillary density could be measured. Similar experiments could also be applied to the study of nerve regeneration in STZ diabetic rats. Cameron *et al.* (1991) found that endoneurial capillary density was not affected by STZ diabetes although prazosin treatment of STZ diabetic rats caused a significant increase in capillary density. Prazosin treatment did not affect capillary density in non-diabetic animals and the aforementioned authors suggested that the reason for this could be because the stimulus required for angiogenesis in nerve involves two factors: both relative tissue hypoxia and the

mechanical stimulus of increased blood flow and capillary pressure on the endothelium of blood vessels. It would be interesting to investigate the effects of prazosin treatment on nerve blood flow and nerve regeneration in STZ diabetic rats.

In conclusion, the blood flow of rat sciatic nerves was reduced after nerve injury and repair and was poorest after muscle grafting. This could possibly be a factor affecting recovery of function after nerve damage. The blood flow of the sciatic nerves of STZ diabetic rats was slightly poorer than the blood flow of the non-diabetic animals, however, it is thought that the small difference in blood flow is unlikely to explain the poorer regeneration rates of diabetic nerve or the poorer recovery of function after nerve injury and repair in STZ diabetic animals. It is also considered that other contributory factors, in addition to the slightly poorer blood flow found in diabetic nerve, are necessary to cause sufficient endoneurial hypoxia to bring about neuropathy.

CHAPTER 9

General Discussion

9. General Discussion.

9.1 Streptozotocin Diabetes as a Model for Diabetic Neuropathy.

It has already been emphasized that nerve morphometry and measurements of electrophysiological indices of nerve conduction in an injured and repaired peripheral nerve do not necessarily give a good indication of functional recovery. In fact, in diabetic nerve where nerve conduction velocity is reduced, there need not be any symptoms of loss of motor or sensory function (Masson and Boulton, 1990). However, the same mechanisms which cause a decrease in conduction velocity in diabetic nerve may also bring about other neuropathic changes which are associated with loss of function, such as axonal degeneration and segmental demyelination.

It has been argued that the reversibility of conduction slowing in nerves of newly diagnosed diabetics and in experimental diabetes may indicate that there is more than one factor operating in the causation of the decrease in conduction velocity (chapter 1.7). It is generally accepted that in human diabetic nerve there is loss of small and large fibres alongside segmental demyelination and remyelination (Greene *et al.*, 1988). Although there has been a report of focal axonopathy in spontaneously diabetic BB Wistar rats (Sima *et al.*, 1990), there have not been any reports of similar axonopathic changes occurring in STZ diabetic rats. These findings suggest that different factors may be operational in causing neuropathic change or, at least, different factors may be predominant in causing neuropathy in diabetic humans as compared to STZ diabetic animals. The finding that an increase in capillary density causes an improvement in the reduced nerve

conduction velocity in STZ diabetic rats but not complete recovery (Maxfield *et al.,* 1993), indicates that a vascular mechanism may not be the only one which causes a reduction in nerve conduction velocity. This would certainly be supported by the findings from experiments presented here (chapter 8) that blood flow in the peripheral nerve of diabetic animals was only reduced by 7%. Unless there had been a concomitant change in vascular permeability or other factor that would increase endoneurial hypoxia, it is unlikely that such a reduction in nerve blood flow could have caused the kind of hypoxia necessary to produce the decrease in conduction velocity recorded in the experiments presented here.

Low *et al.* (1984) believed that the decrease in conduction velocity was likely to have been caused by a decrease in axonal diameter, however, the early decrease in conduction velocity that is found in STZ diabetic rats is inconsistent with this theory. Jakobsen (1979) noted a 3 to 4% reduction in nerve conduction velocity by 24 to 36 hours after STZ administration. It was found in the work presented here that there was a diminution in the size of axon and fibre diameters measured from rats in the diabetic control group as compared to non-diabetic controls although the difference was not significant ($p > 0.05$). It was argued that the smaller diameters measured in the diabetic animals could have been caused by greater shrinkage of the nerve tissue during processing because of the greater tissue osmolarity (see chapter 4.4.4). This could be tested in future studies comparing the fibre and axon diameters, alongside measurements of tissue osmolarity, in short-term and long-term STZ diabetic rats. However, it is unlikely that the small decrease in axon and fibre diameter could have caused the large decrease in conduction velocity that was recorded from the diabetic animals in the work presented in this thesis.

Therefore, if the decrease in nerve conduction velocity in STZ diabetic rats was not caused by a decrease in fibre or axon diameter and is unlikely to have been accounted for by energy deprivation through endoneurial hypoxia; what mechanism could be operating to cause the reduction in conduction velocity ? Greene *et al.* (1988) proposed a mechanism by which an increase in polyol metabolism and a concomitant decrease in tissue myoinsitol could result in a disruption of Na^+/K^+ ATP-ase function, thus producing a change in endoneurial Na^+ concentration and a decrease in nerve conduction velocity (see chapter 1.7, metabolic hypothesis). The irreversibilty of the decrease in conduction velocity in long-term diabetes could be explained by the migration of paranodal axo-glial junctional complexes that was observed by Sima *et al.* (1985) in spontaneously diabetic BB Wistar rats. These authors theorized that the migration of complexes would result in a change in Na^+ permeability which could cause an irreversible decrease in conduction velocity (chapter 4.1.6).

From the evidence presented here and from the work of other authors, it would seem probable that the predominating mechanism behind the changes observed in peripheral nerve of STZ diabetic animals is of a metabolic nature rather than vascular. This is not necessarily true of human diabetic nerve where the aetiology of focal neuropathies would seem to be better explained by a vascular mechanism. It is conceivable, however, that the differences in the presentation of neuropathy in human diabetes and STZ diabetic rats could be accounted for by anatomical differences. It has been suggested that long nerves are more susceptible to multifocal injuries which would explain the vulnerability of longer axons in human diabetes (Thomas, 1990). In animal models of diabetes where the lengths of nerves are considerably shorter, the loss of axons would be expected to be smaller. An alternative view is that a decrease in perikaryal synthesis and axonal

transport would result in the inability of the cell to maintain fibres in the periphery; hence, the prevalence of distal symmetric neuropathy could be explained. The loss of fibres in animal models would be expected to be smaller than in humans because of the difference in length of axons.

Alternatively, the difference between human diabetes and STZ diabetic rats in terms of neuropathic change could be because of the difference in the duration of the diabetic state. Studies involving animals tend to be relatively short in comparison to the duration of the disease in patients with neuropathy. In addition to metabolic factors, the duration of the disease in humans makes the possibility of vascular disease more probable. The rats used in the experiments described here had been diabetic for around 150 days but statistical differences in fibre and axon diameter were not reported. If the duration of the diabetic state is the most influential factor in determining the appearance of axonopathy, perhaps 150 days was not long enough to have observed such pathology or more likely, STZ diabetic rats are not vulnerable to the same vascular changes as humans (Tomlinson *et al.*, 1992).

In conclusion, if the predominant mechanism causing changes in the peripheral nerve of STZ diabetic rats is different from that operating in humans, the role of STZ diabetic rats as a model for human diabetes should be considered carefully.

9.2 Peripheral Nerve Regeneration in Diabetes.

It was found from the experiments presented in chapter 4 that the axon and fibre diameters in the diabetic crush group were significantly smaller than in the non-diabetic animals ($p < 0.01$). It has been argued that this finding demonstrates a poorer recovery of fibre and axon diameter in diabetics and that the absence of a significant difference in the

nerve-to-nerve suture, nerve graft and muscle graft groups was probably because the recovery of fibre size in the non-diabetic animals in these groups was also poor. Thus, the difference between diabetic and non-diabetic animals was too small to be significant (chapter 4.4.4). The results from the experiments of chapter 8 showed that there was no significant difference in the blood flow through the repaired sciatic nerves of diabetic and non-diabetic animals. This result indicates that the difference in recovery after nerve repair between non-diabetic and diabetic animals was unlikely to have been caused by poorer blood flow in the latter. The findings of other authors that the capacity for diabetic nerve to regenerate is poorer than non-diabetic nerve (Longo *et al.*, 1986; Ekström *et al.*, 1989; Ekström & Tomlinson, 1989) and the findings of poorer recovery of fibre and axon diameter in peripheral nerve of STZ diabetic rats used in the experiments presented here, could both be explained by a decrease in axonal transport. McLean *et al.* (1987) demonstrated that there was impairment in the induction of the enzyme ODC in the dorsal root ganglia of STZ diabetic rats after nerve crush. These authors argued that the subsequent protein synthesis and axonal transport of synthesized substances would necessarily be reduced and, therefore, could result in a reduced rate of regeneration. It has been suggested that the signal to the cell body could be retrogradely transported NGF, IGF-I or insulin (Kanje *et al.*, 1986). Poor retrograde transport of NGF and insulin deficiency are features of STZ diabetes (Jakobsen *et al.*, 1981) and NGF and insulin have both been found to increase ODC activity (Russell, 1985).

The relevance of the recovery of axon and fibre diameter to the recovery of function after nerve injury and repair is not direct, however, a decreased rate of regeneration could affect recovery of function in a number of ways. Shrinkage of endoneurial tubes has been found to progress over

time (Seddon, 1972) and a slower rate of regeneration could result in shrunken endoneurial tubes providing hinderence to the passage of regenerating axons and their subsequent maturation (Simpson & Young, 1945). Also, a slower rate of regeneration allows longer time for the degeneration of distal endoneurial tubes, thus removing axonal guidance for pioneering axons.

If the reason for a reduced rate of regeneration in diabetic nerve were a decrease in axonal transport, it might be expected that recovery would be poorer in all fibre types. Interestingly, a significant decrease in the recovery of peroneal cutaneous receptive field area was found in the diabetic muscle graft group ($p < 0.01$) and recovery was poorer in all diabetic groups as compared to like groups of non-diabetic animals (see chapter 5.3); whereas, no significant difference was found in the recovery of motor function between non-diabetic and diabetic animals. It was discussed in chapter 6.4.1 that the smaller tensions produced by the diabetic animals were probably attributable to the smaller size of the soleus muscle in the diabetic animals. In order to confirm this suggestion, further studies could be performed on weight-matched non-diabetic and diabetic animals to investigate the tensions produced by the soleus muscle after nerve injury and repair. However, the design of such experiments would be particularly difficult because diabetic rats do not gain weight to the same degree as non-diabetic animals (see chapter 3.3.2). Perhaps, therefore, the best way do deal with this problem would be to find the correlation between the mass or the cross-sectional area of the soleus muscle (chapter 6.1.5) and the tension produced in the same muscle, for large numbers of both diabetic and non-diabetic animals after nerve injury and repair.

The finding of the work presented here that recovery of function after nerve injury and repair was only significantly worse in diabetic rats in the

sensory system is fascinating considering that in diabetic neuropathy it is sensory nerves that are more frequently affected. The reason for poorer recovery of function of sensory nerves has been discussed in chapter 5.4.2. Possibly abnormal NGF and/or abnormal transport of NGF could cause a decreased capacity of peripheral sensory axons to regenerate by the mechanism described briefly above (see chapter 1.8). Lunn *et al.,* (1991) found that the poor recruitment of macrophages to the site of Wallerian degeneration in C57BL/Ola mice and the consequent reduction in NGF could be the cause of the poor regeneration of sensory axons observed in these mice. Brown *et al.* (1991) found that injecting NGF into the saphenous nerve improved nerve regeneration. They have found evidence that motor and sensory axons differ in their trophic requirements.

In conclusion, it has been demonstrated that in STZ diabetic rats there is poorer recovery of axon and fibre diameter which may be due to a decreased rate of regeneration. The difference in the recovery between non-diabetic and diabetic animals is unlikely to be because of differences in blood supply. This suggestion is substantiated by the fact that the recovery of function after nerve injury and repair in STZ diabetic rats is not general but appears to be poorer in the return of sensation. The selectively poorer recovery of sensation in the STZ diabetic rats used in the experiments presented here may be explained by a decreased production of NGF and/or a decreased rate of retrograde transport of NGF which subsequently results in a poorer rate of regeneration. In retrospect, experiments of shorter duration (i.e. 50 to 100 days) would probably have been preferable in order to show differences in recovery of diabetic and non-diabetic animals after nerve repair because of the similarity in the recovery of function in rats by 150 days.

9.3 Peripheral Nerve Regeneration.

From a comparison of the recovery of function between different methods of nerve injury and repair, the recovery was consistently better after nerve crush injury than after injuries of the neurotmesis type and subsequent repair. Many comparisons have been made between different types of nerve injury and repair by other researchers working in the field of nerve regeneration and the findings presented here that recovery was best after injury of the axonotmesis type corroborates their findings (Gutmann *et al.*, 1942; Cragg & Thomas, 1964; Hyde & Scott, 1983). It is generally accepted that the reason for better recovery after injuries of the axonotmesis type (Sunderland type II) is that the endoneurial tubes remain in continuity after nerve crush injury (Thomas, 1964) and provide contact guidance for regenerating axons to their original end organs.

The findings from the work described here demonstrated that of the other three types of operated group, the recovery was generally best after nerve-to-nerve suture and poorest after muscle graft. Types of nerve repair have already been discussed in chapter 1.3. In general, one suture line is considered better than two when repairing peripheral nerve, however, where tension on the nerve after repair is great, it may be preferable to use a nerve graft with two suture lines and reduce the tension (Millesi & Meissl, 1981). The reasons for the poorer regeneration through two suture lines could be because of greater production of collagen and endoneurial scarring. In addition to the scar tissue providing impedance to regenerating axon sprouts and preventing them from reaching target tissue, two suture lines may produce greater cross-wiring of regenerating axons because the regenerating axons have to overcome two barriers resulting in a greater mis-match of fibres in the distal stump. Seddon (1972) suggested that

poorer regeneration through two suture lines was inevitable because however good the surgeon is, each suture line is an injury in itself and will be followed by an inflammatory reaction. Oedema can distort the stroma of the nerve and aggravate the irregularity of the pattern of regeneration.

One argument that might explain the poorer recovery after muscle grafts is that adhesion between the graft and the surrounding tissue could put harmful traction on the nerve. In the experiments described here, it was commonly found that separation of the muscle graft from the surrounding tissue was difficult. It also possible that a smaller difference was found in the recovery of function after muscle grafting than after nerve grafting because there was tension on the nerve. Severance and suture of the sciatic nerve in two places inevitably produced more tension on the nerve than was present before the injury. Tension on the nerve, could restrict blood flow and result in poorer regeneration (Millesi & Meissl, 1981). Recovery could have been poorer after muscle grafting than after other types of repair because of the lack of Schwann cells in the graft and the need for these cells to migrate a further distance to reach the proximal stump. Recovery after nerve grafting would be expected to be better because Schwann cells are already present in the graft. Among other roles, Schwann cells produce NGF. Interestingly, the recovery of peroneal nerve cutaneous receptive field was extremely poor after muscle grafting in the diabetic animals. The possible importance of NGF in the regeneration of sensory nerve in diabetes has been discussed above. It is plausible that muscle grafting and the lack of Schwann cells could exacerbate an already poor situation for the regeneration of sensory fibres. In addition, the experiments described in chapter 8 demonstrated that the recovery of nerve blood supply did not return to normal after any type of injury and repair and was poorest after muscle grafting. It is possible that regeneration after muscle grafting could

be poorer than after other methods of nerve injury and repair because of a difference in the capillarization of muscle graft tissue as compared to nerve tissue.

Although the recovery after muscle grafting tended to be poorer than after nerve grafting, the two types of recovery were only significantly different in the recovery of sensation in the diabetic animals. Therefore, nerve grafts are almost certainly preferable to muscle grafts when the use of the former is practicable. When this is not the case, muscle grafts undoubtedly provide a natural alternative means of repair, for example in the treatment of painful neuromata and digital nerve injuries.

Unfortunately, the results from the work described in chapter 7 demonstrated that after injuries of the neurotmesis type, proper function of the stretch reflex does not return. It has been inferred that the failure of the stretch reflex to return is probably due to a combination of a decreased number of functional reconnections having been made and to the formation of inappropriate ones. The degree of cross-wiring of regenerating axons and the resulting aberrant innervation must be the most important and impossible barrier to the restoration of function after nerve injury and repair. It would seem, therefore, that however much the present day scientist knows about the anatomy and physiology of the peripheral nervous system, however good the surgeon is and whatever method of repair he choses, he or she will depend, ultimately, on forces governed by randomness and probability for maximal structural and functional recovery after the repair of the peripheral nerve.

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Appendix 1.

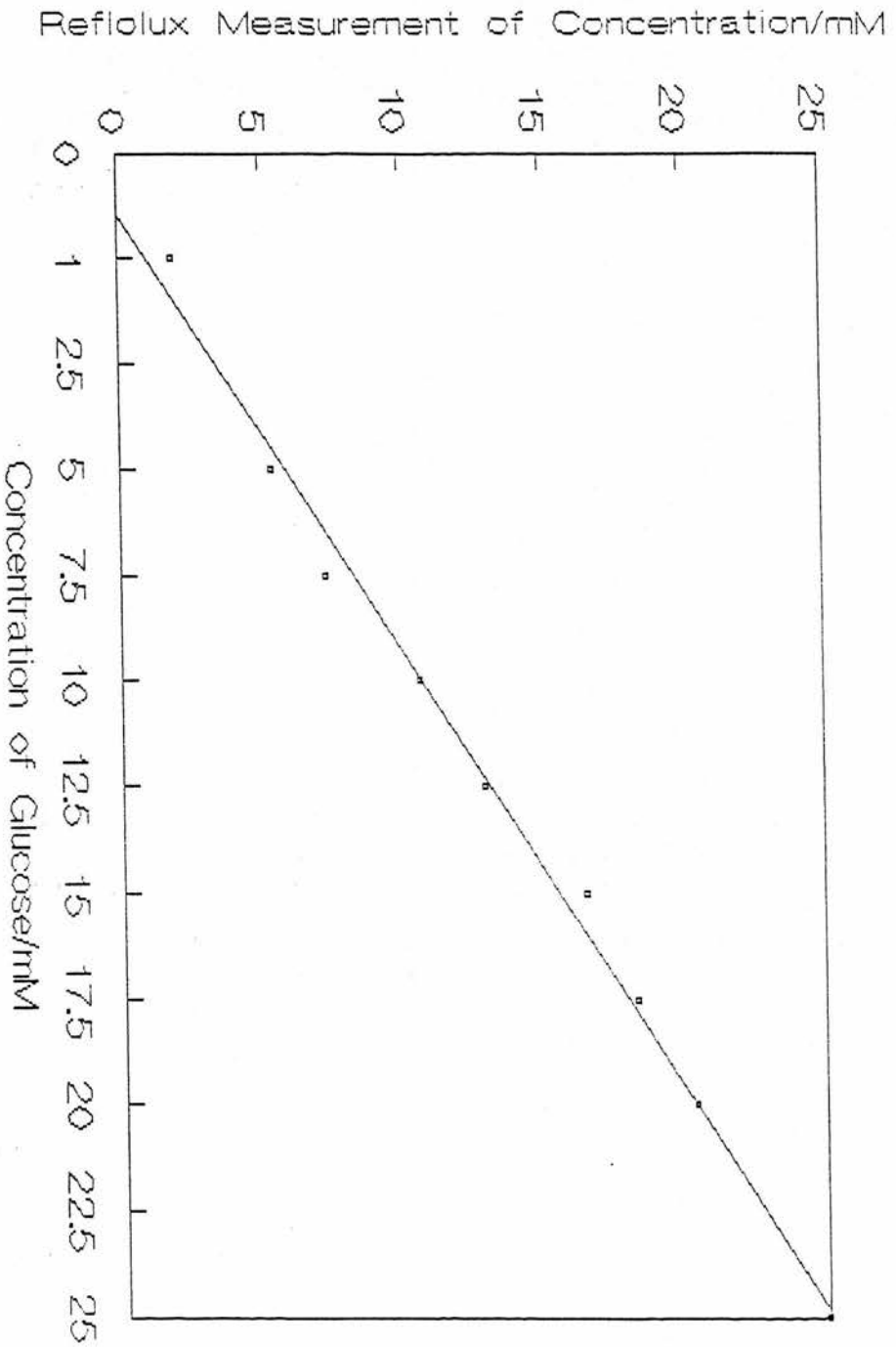
The composition of the diet given to the rats used in the work presented in this thesis and the composition of a normal small animal diet

Normal Diet	Modified Maintenance Diet
oil 3%	oil 2.6%
protein 18%	protein 14.6%
fibre 4%	fibre 4.3%
ash 5%	ash 5.8%
nitrogen-free extract 60%	nitrogen-free extract 62.7%

The rats used in the experiments presented in this thesis were given the modified maintenance diet, which is the recommended food substance produced by Special Dietary Supplements (SDS, Essex, U.K.) for use in long-term experiments.

Appendix 2.

Measurements of glucose concentration recorded by the Reflolux S reflectance photometer plotted against the concentration of glucose in standard solutions



Appendix 3.

Methods to prepare the solutions required for fixing and processing nerve tissue

To prepare 0.4 M sodium cacodylate solution.

Dissolve 8.561 g of sodium cacodylate in 50 ml of distilled water. Add distilled water to make up to 100 ml.

To prepare 0.2 M hydrochloric acid.

Add 20 ml of 1 M hydrochloric acid to 80 ml of distilled water to give a 0.2 M solution.

To prepare 0.2 M sodium cacodylate buffer, pH 7.4.

To 50 ml of 0.4 M sodium cacodylate add approximately 8 ml of 0.2 M hydrochloric acid to give a pH of the solution of 7.4. Add distilled water to make up to 100 ml.

To prepare a 4% solution of glutaraldehyde in

0.1 M sodium cacodylate buffer.

Add 50 ml of 0.2 M sodium cacodylate buffer (pH = 7.4) to 16 ml of 25% glutaraldehyde. Add 0.2 M hydrochloric acid, if necessary, to bring the pH to 7.4. Add distilled water to make up to 100 ml.

Appendix 4.

Methods to prepare the solutions required for processing of nerve tissue

To prepare 5% sucrose buffer solution.

Add 5 g of sucrose, slowly, to 50 ml of 0.2 M of sodium cacodylate buffer, stirring continuously. Add distilled water to make up to 10 ml.

To prepare a solution of 0.1% osmium tetroxide

in 0.1 M sodium cacodylate buffer.

Break an ampoule containing 0.1 g of osmium teroxide in a sealed universal bottle, in a fume cupboard. Add 10 ml of 0.1 M sodium cacodylate buffer (pH = 7.4). Mix the solution and leave overnight to ensure that all of the compound dissolves.

Appendix 5.

Method to prepare TESPA (3-aminopropyltriethoxyxylane) coated slides

(1) Wash slides in soapy water and rinse in tap water.

(2) Rinse slides in 95% alcohol and 0.1% glacial acetic acid.

(3) Dip the slides in2% TESPA(3-aminopropyltriethoxyxylane; Sigma Chemical Company Ltd, Poole, U.K.) in acetone. Dry the slides at room temperature in a fume cupboard.

(4) Dip the slides in acetone twice, each time for 10 s, and dry the slides in an oven at 37^{o}C.

Appendix 6.

Methods to prepare solutions required for immunocytochemistry on rat pancreas

To prepare 0.5 M tris solution.

Dissolve 30.25 g of trisma base in 500 ml of distilled water.

To prepare normal hydrochloric acid.

Mix 85 ml of concentrated hydrochloric acid with 1000 ml of distilled water.

To prepare normal saline.

Dissolve 42.5 g of sodium chloride in 5000 ml of distilled water.

To prepare tris-buffered saline (TBS).

Add normal hydrochloric acid to 500 ml of 0.5 M tris solution until pH is 7.6. Add distilled water to make up to 2000 ml. Dilute in a 1:10 ratio with normal saline for use.

To prepare 0.2 M tris solution.

Dissolve 24.28 g of trisma base in 1000 ml of distilled water.

To prepare 0.1 normal hydrochloric acid.

Add 8.5 ml of concentrated hydrochloric acid to 500 ml of distilled water. Make up to 1000 ml with distilled water.

To prepare diaminobenzidine (DAB) buffer.

Mix 24 ml of 0.2 M tris solution to 38 ml of 0.1 normal hydrochloric acid. Dissolve 0.068 g of imidazole.d 38 ml of distilled water to make up to 100 ml.

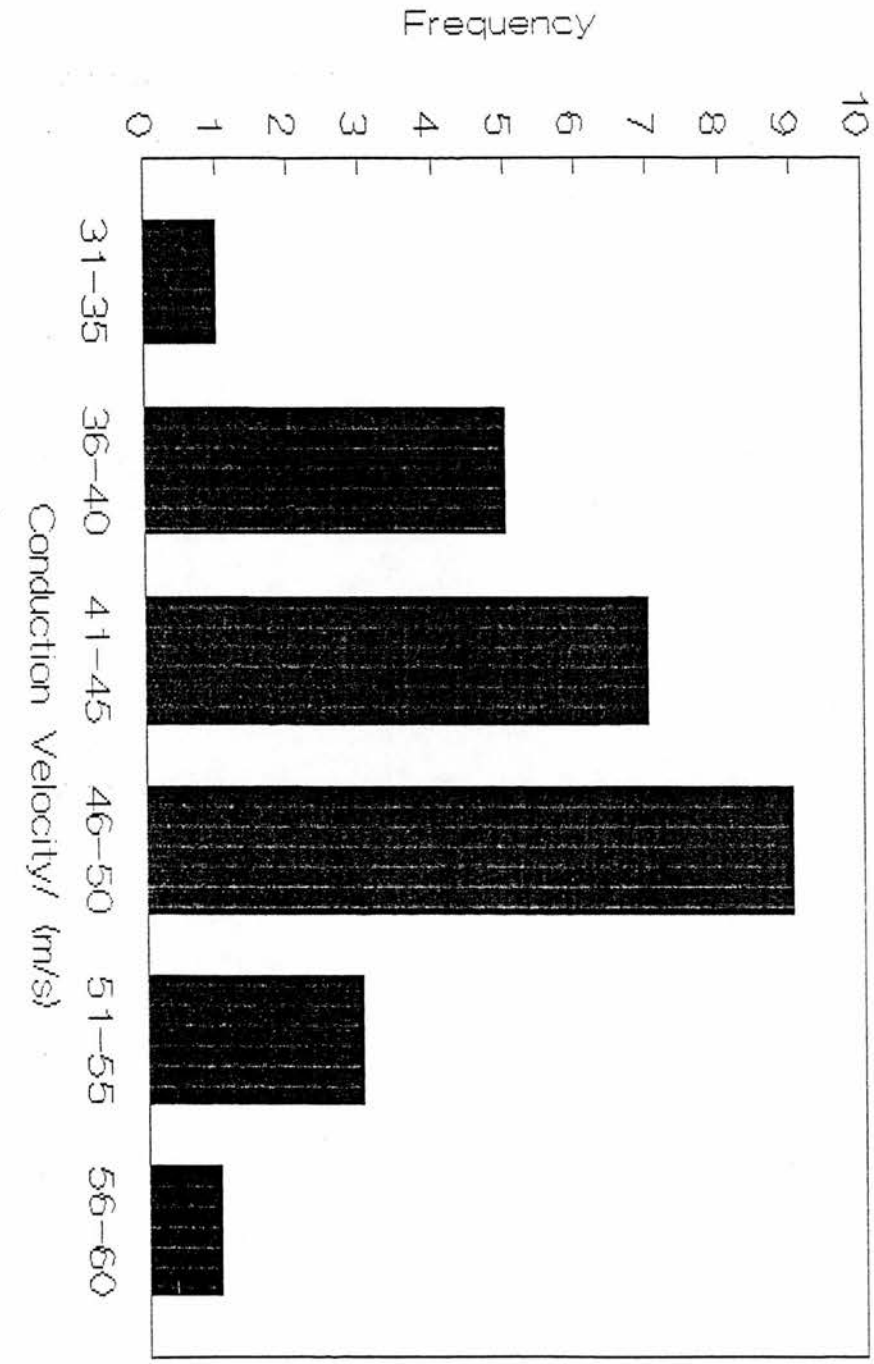
Appendix 7.

Values of conduction velocity of left/operated sciatic nerves,right unoperated sciaitic nerves and CV ratio for the rats used in the experiments presented in chapter 4 (2DP)

RAT GROUP	left CV /m s^{-1}	rightCV /m s-1	CV RATIO	RAT GROUP	left CV /m s-1	rightCV /m s^{-1}	CV RATIO
C	48.48	46.30	1.05	DC	36.72	47.60	0.77
	38.67	38.67	1.00		18.90	18.89	1.00
	46.51	30.88	1.51		38.10	34.68	1.10
	46.83	43.75	1.07		20.39	24.29	0.84
	43.59	47.89	0.91		22.19	25.78	0.86
CR	36.81	43.48	0.85	DCR	22.79	27.73	0.82
	32.78	47.83	0.69		17.31	25.69	0.67
	27.35	49.23	0.56		27.42	35.42	0.77
	39.22	39.23	1.00		18.23	29.30	0.62
	27.23	44.94	0.61		24.24	29.31	0.83
NN	33.04	45.95	0.72	DNN	18.33	20.00	0.92
	33.96	47.35	0.72		19.02	18.06	1.05
	39.29	51.56	0.76		19.50	25.00	0.78
	56.67	56.00	1.01		16.44	28.05	0.59
	42.26	45.71	0.92		19.32	24.77	0.78
NG	36.54	46.52	0.79	DNG	18.52	41.86	0.44
	27.59	39.29	0.70		19.44	28.53	0.68
	19.65	42.39	0.46		7.98	20.83	0.38
	18.50	54.84	0.34		8.24	15.56	0.53
	5.96	40.91	0.15		13.16	22.73	0.58
MG	14.30	46.52	0.31	DMG	8.01	24.24	0.33
	10.78	39.29	0.27		3.56	11.11	0.32
	15.36	42.39	0.36		10.94	21.58	0.51
	9.68	54.84	0.18		4.21	30.83	0.14
	16.75	36.60	0.46		4.00	18.18	0.22
	7.15	40.91	0.17		7.18	39.00	0.18

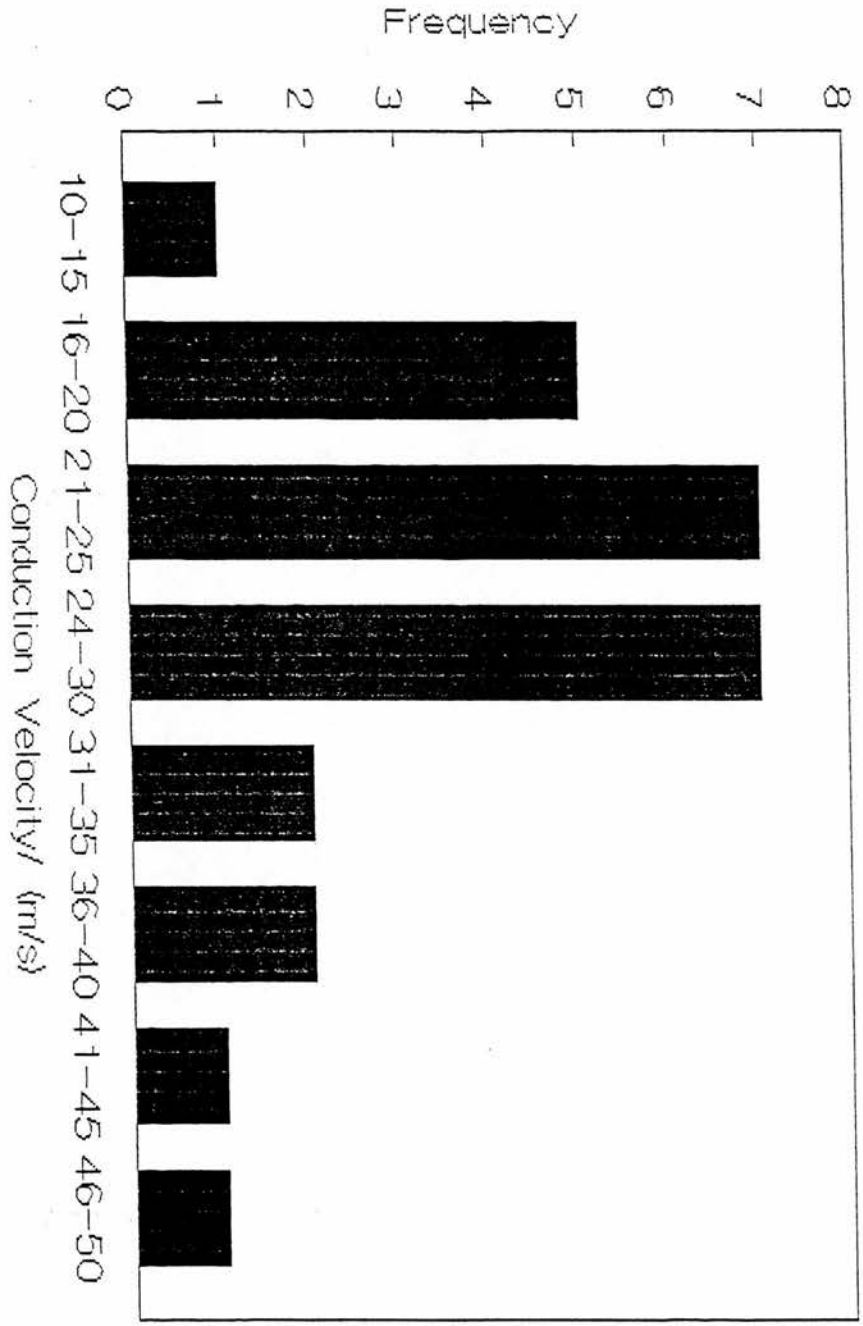
Appendix 8.

Frequency histogram of the values of conduction velocity of right/unoperated sciatic nerves for the non-diabetic rats used in the experiments described in chapter 4



Appendix 9.

Frequency histogram of the values of conduction velocity of right/unoperated sciatic nerves for the diabetic rats used in the experiments described chapter 4



Appendix 10.

Values of chronaxie for left/operated and right/unoperated sciatic nerves for the rats used in the experiments described in chapter 4 (2DP)

RAT GROUP	LEFT /ms	RIGHT /ms	RAT GROUP	LEFT /ms	RIGHT /ms
C	0.16	0.21	DC	0.23	0.16
	0.13	0.78		0.17	0.11
	0.26	0.37		0.24	0.16
	0.46	0.28		0.12	0.16
	0.42	0.24		0.17	0.26
CR	0.27	0.66	DCR	0.18	0.18
	0.22	0.34		0.09	0.27
	0.45	0.41		0.16	0.14
	0.5	0.15		0.3	0.22
	0.23	0.45		0.23	0.5
NN	0.27	0.28	DNN	0.1	0.32
	0.24	0.24		0.19	0.36
	0.18	0.19		0.3	0.27
	0.36	0.35		0.15	0.46
	0.33	0.26		0.37	0.28
NG	0.33	0.91	DNG	0.26	0.2
	0.17	0.2		0.12	0.08
	0.07	0.08		0.14	0.13
	0.34	0.1		0.14	0.1
	0.2	0.3		0.3	0.19
MG	0.16	0.13	DMG	0.22	0.2
	0.2	0.19		0.28	0.13
	0.43	0.18		0.74	0.45
	0.1	0.08		0.5	0.48
	0.34	0.21		0.27	0.14
				0.22	0.13

Appendix 11.

Values of fibre and axon diameter, G-ratio and myelin thickness in the non-diabetic animals used in the experiments described in chapter 4 (3DP)

RAT GROUP	FIBRE /μm	AXON /μm	G RATIO	MYELIN /μm
C	7.470	4.211	0.548	1.629
	7.534	4.199	0.548	1.667
	7.214	3.564	0.481	1.825
	7.214	4.188	0.567	1.514
	7.329	4.202	0.557	1.564
CR	4.782	3.009	0.605	0.886
	5.567	2.836	0.489	1.366
	5.669	2.868	0.489	1.401
	6.283	2.887	0.447	1.698
	6.040	2.564	0.424	1.738
NN	4.284	1.941	0.418	1.172
	4.399	1.902	0.406	1.248
	3.405	1.797	0.531	0.804
	3.975	2.152	0.514	0.912
	4.023	1.883	0.450	1.070
NG	4.080	1.581	0.371	1.249
	4.151	1.648	0.376	1.252
	3.902	1.865	0.464	1.019
	3.694	1.771	0.464	0.962
	3.882	1.833	0.460	1.025
MG	3.857	1.641	0.400	1.108
	4.013	1.967	0.521	1.023
	3.946	1.555	0.380	1.195
	3.770	1.502	0.378	1.134
	3.336	1.237	0.365	1.050

Appendix 12.

Values of fibre and axon diameter, G-ratio and myelin thickness in the diabetic animals used in the experiments described in chapter 4 (3DP)

RAT GROUP	FIBRE /μm	AXON /μm	G RATIO	MYELIN /μm
DC	6.566	3.560	0.532	1.500
	6.804	3.623	0.510	1.591
	6.935	4.103	0.580	1.416
	7.536	4.043	0.527	1.747
	7.119	3.99	0.549	1.564
DCR	5.089	2.490	0.473	1.300
	4.742	2.293	0.464	1.224
	4.466	1.803	0.386	1.331
	4.303	1.730	0.371	1.286
	4.533	2.209	0.472	1.162
DNN	4.641	2.216	0.465	1.212
	3.737	2.046	0.531	0.846
	3.600	1.367	0.370	1.116
	4.570	2.408	0.514	1.081
	4.172	1.876	0.423	1.149
DNG	4.680	1.899	0.378	1.391
	3.861	1.454	0.362	1.204
	3.462	1.623	0.456	0.919
	4.079	1.970	0.467	1.054
	3.636	1.384	0.363	1.126
DMG	3.106	1.304	0.405	0.901
	3.389	1.635	0.464	0.877
	3.519	1.456	0.399	1.031
	3.196	1.155	0.356	1.020
	3.670	1.709	0.450	0.981

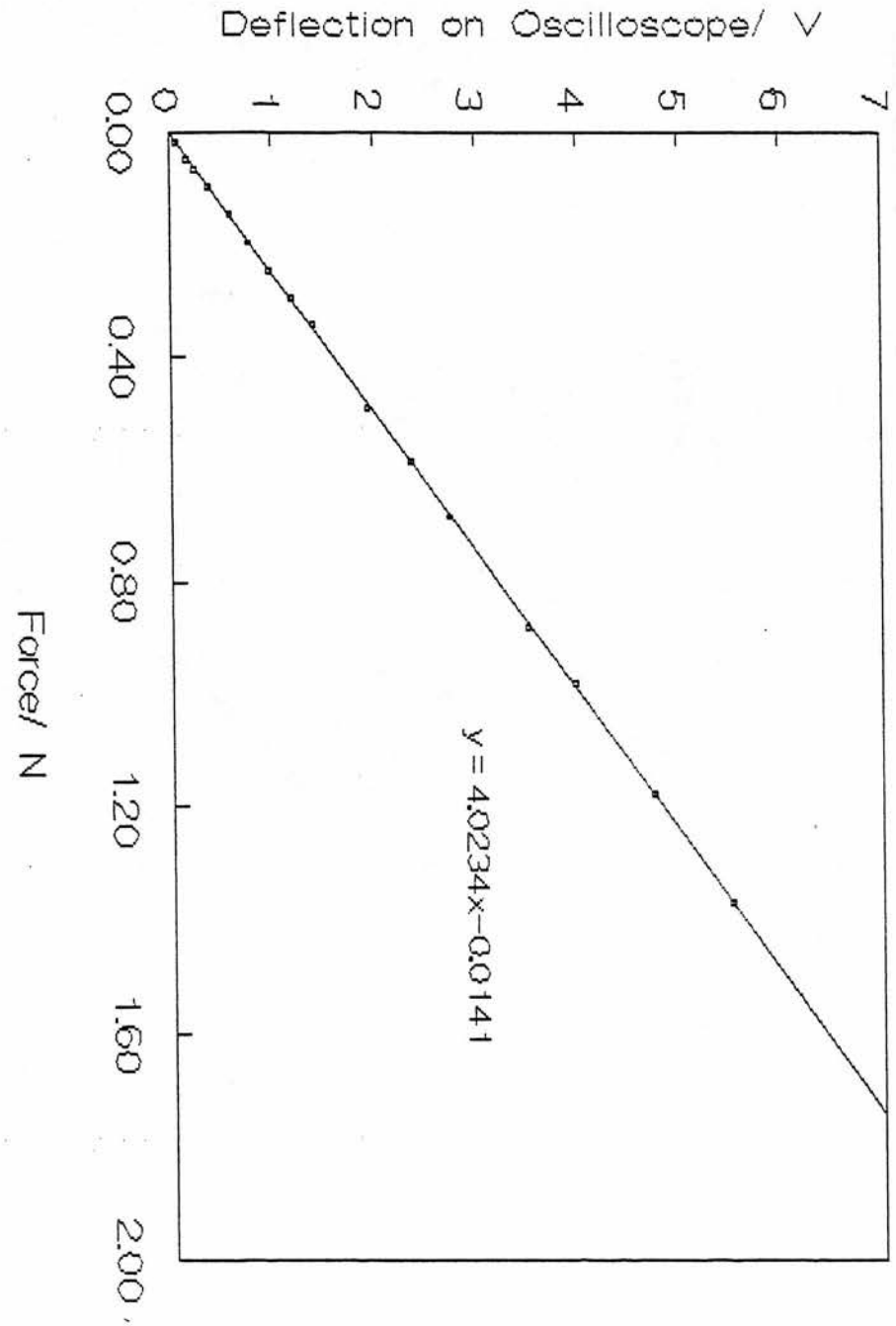
Appendix 13.

Values of left and right peroneal nerve cutaneous receptive fields, and the ratio of left/right (2DP)

RAT GROUP	RIGHT RF /cm$^{2)}$	LEFT RF /cm2)	RF RATIO	RAT GROUP	RIGHT RF /cm2)	LEFT RF /cm2	RF RATIO
C	8.16	10.54	1.29	DC	8.92	9.09	1.02
	9.09	10.56	1.16		9.59	10.21	1.06
	11.79	11.60	0.98		10.25	8.38	0.82
	10.38	8.20	0.79		9.32	10.06	1.08
	11.27	9.99	0.89		9.57	9.96	1.04
CR	10.16	7.03	0.69	DCR	7.55	7.28	0.96
	10.36	8.08	0.78		9.10	6.18	0.68
	9.36	7.91	0.85		10.00	9.14	0.91
	8.82	10.46	1.19		8.22	7.14	0.87
	8.08	8.00	0.99		9.29	6.31	0.68
NN	7.28	4.98	0.68	DNN	8.29	5.01	0.60
	9.81	9.20	0.94		10.35	9.67	0.93
	9.32	7.59	0.81		9.55	7.65	0.80
	8.24	9.99	1.21		7.15	5.80	0.81
	9.09	8.37	0.92		9.44	8.05	0.85
NG	9.83	9.41	0.96	DNG	8.52	5.86	0.69
	11.24	8.17	0.73		7.79	6.26	0.80
	10.78	6.60	0.61		8.69	0.00	0.00
	11.23	8.06	0.72		7.71	3.99	0.52
	8.20	6.40	0.78		9.93	6.93	0.70
	11.05	9.10	0.82				
	9.51	6.48	0.68				
MG	14.11	9.69	0.69	DMG	7.27	2.37	0.33
	11.01	10.88	0.99		10.69	5.00	0.47
	11.60	7.06	0.61		8.10	0.00	0.00
	10.60	9.57	0.90		7.59	0.00	0.00
	10.86	9.72	0.90		9.32	4.50	0.48

Appendix 14.

Calibration curve for the #52-9503 isometric tension transducer.



Appendix 15.

Values of the time to peak tension, time to half peak tension, time to half relaxation, duration of twitch, time-tension integral and time-tension index of the isometric twitch (indirect stimulation) in non-diabetic (3DP)

	F /N	t /s	$t_{1/2}$ /s	$r_{1/2}$ /s	t_d /s	TT /N s	TTI /N
C	1.187	0.047	0.027	0.021	0.255	0.219	0.859
	0.543	0.058	0.035	0.023	0.242	0.114	0.471
	0.873	0.044	0.025	0.025	0.361	0.223	0.618
	1.395	0.049	0.031	0.021	0.214	0.257	1.201
	1.378	0.051	0.030	0.021	0.204	0.241	1.181
CR	1.030	0.047	0.026	0.023	0.246	0.211	0.858
	0.682	0.048	0.029	0.019	0.166	0.166	1.000
	0.682	0.043	0.023	0.027	0.212	0.144	0.679
	0.943	0.056	0.035	0.023	0.347	0.226	0.651
	1.082	0.037	0.020	0.024	0.342	0.263	0.769
	0.448	0.044	0.027	0.024	0.350	0.087	0.249
	0.264	0.040	0.025	0.034	0.234	0.09	0.385
NN	0.230	0.038	0.021	0.026	0.225	0.061	0.271
	0.160	0.026	0.015	0.024	0.135	0.034	0.252
	0.247	0.042	0.03	0.013	0.240	0.023	0.096
	0.069	0.043	0.021	0.031	0.233	0.018	0.077
	0.351	0.045	0.026	0.020	0.297	0.060	0.202
	0.404	0.047	0.030	0.023	0.277	0.0842	0.304
	0.351	0.048	0.030	0.012	0.210	0.040	0.190
	0.257	0.046	0.031	0.016	0.193	0.033	0.171
	0.2826	0.040	0.025	0.014	0.187	0.037	0.198
	0.346	0.044	0.031	0.008	0.123	0.020	0.163
NG	0.158	0.109	0.020	0.245	0.939	0.134	0.143
	0.473	0.038	0.014	0.038	0.192	0.013	0.068
	0.177	0.026	0.015	0.024	0.130	0.033	0.254
	0.809	0.042	0.020	0.028	0.312	0.056	0.179
	0.177	0.066	0.032	0.096	0.322	0.097	0.301
MG	1.023	0.054	0.032	0.026	0.487	0.103	0.211
	0.104	0.039	0.016	0.062	0.485	0.059	0.122
	0.309	0.053	0.023	0.045	0.405	0.053	0.131
	0.197	0.049	0.019	0.040	0.327	0.073	0.223
	0.210	0.057	0.021	0.034	0.348	0.051	0.147

Values of the time to peak tension, time to half peak tension etc. from muscle twitch experiments (indirect stimulation) in diabetic animals (3DP)

	F /N	t /s	$t_{1/2}$ /s	$r_{1/2}$ /s	t_d /s	TT /N s	TTI /N
DC	0.134	0.066	0.020	0.154	0.210	0.135	0.643
	0.414	0.053	0.031	0.025	0.241	0.104	0.432
	1.057	0.048	0.032	0.022	0.273	0.179	0.656
	0.752	0.057	0.036	0.024	0.363	0.167	0.460
	0.796	0.054	0.035	0.022	0.457	0.16	0.350
	0.369	0.056	0.036	0.024	0.201	0.087	0.433
	0.466	0.054	0.034	0.022	0.323	0.145	0.449
	0.431	0.054	0.034	0.024	0.261	0.14	0.536
DCR	0.327	0.047	0.032	0.034	0.545	0.201	0.369
	0.438	0.042	0.026	0.021	0.148	0.088	0.595
	0.111	0.047	0.028	0.020	0.292	0.197	0.675
	0.752	0.052	0.033	0.027	0.122	0.148	1.213
	0.456	0.044	0.026	0.025	0.390	0.128	0.328
	0.901	0.043	0.024	0.021	0.772	0.302	0.391
	0.329	0.049	0.032	0.210	0.484	0.068	0.140
	0.130	0.058	0.027	0.129	0.860	0.128	0.149
	0.501	0.053	0.033	0.023	0.815	0.227	0.279
DNN	0.031	0.035	0.022	0.009	0.123	0.003	0.024
	0.199	0.046	0.023	0.026	0.17	0.041	0.241
	0.277	0.041	0.026	0.018	0.225	0.056	0.249
	0.247	0.053	0.034	0.027	0.219	0.051	0.233
	0.309	0.06	0.044	0.026	0.295	0.066	0.224
	0.396	0.055	0.029	0.032	0.347	0.12	0.346
	0.369	0.053	0.034	0.025	0.611	0.129	0.211
	0.451	0.053	0.033	-	0.077	0.177	2.299
DNG	0.070	0.041	0.025	0.113	0.678	0.053	0.078
	0.391	0.047	0.022	0.033	0.334	0.119	0.356
	0.317	0.039	0.027	0.026	0.342	0.085	0.249
	0.543	0.047	0.028	0.030	0.191	0.112	0.586
	0.327	0.047	0.03	0.029	0.672	0.106	0.158
	0.220	0.056	0.027	0.057	0.359	0.100	0.279
	0.247	0.057	0.026	0.053	0.423	0.114	0.270
DMG	0.160	0.044	0.022	0.050	0.472	0.074	0.157
	0.182	0.043	0.070	0.029	0.439	0.072	0.164
	0.135	0.044	0.020	0.036	0.350	0.065	0.186
	0.124	0.047	0.026	0.038	0.293	0.080	0.273
	0.381	0.041	0.022	0.027	0.475	0.108	0.227

Appendix 16.

Values of peak tension, time to peak tension, time to half peak tension, time to half relaxation, from the time to peak tension, duration, time-tension integral and time-tension index of the isometric twitch (direct stimulation) in non-diabetic (3DP)

	F /N	t /s	$t_{1/2}$ /s	$r_{1/2}$ /s	t_d /s	TT /N s	TTI /N
C	0.264	0.080	0.033	0.099	0.583	0.189	0.324
	0.299	0.091	0.031	0.130	0.693	0.264	0.381
	0.274	0.08	0.031	0.105	0.591	0.216	0.365
	0.143	0.055	0.024	0.080	0.422	0.074	0.175
	0.292	0.082	0.028	0.100	0.703	0.228	0.324
CR	0.264	0.097	0.033	0.134	0.661	0.250	0.378
	0.150	0.089	0.034	0.200	0.768	0.186	0.242
	0.150	0.070	0.030	0.093	0.705	0.134	0.190
	0.133	0.068	0.029	0.123	0.585	0.106	0.181
	0.125	0.056	0.021	0.056	0.462	0.064	0.139
	0.0781	0.065	0.024	0.071	0.219	0.036	0.164
	0.098	0.103	0.044	0.355	0.887	0.174	0.196
NN	0.098	0.060	0.028	0.127	0.587	0.085	0.145
	0.078	0.070	0.029	0.210	0.743	0.104	0.140
	0.182	0.058	0.022	0.177	0.713	0.180	0.252
	0.138	0.071	0.029	0.153	0.708	0.127	0.179
	0.156	0.058	0.022	0.098	0.559	0.104	0.186
	0.160	0.048	0.022	0.076	0.399	0.088	0.221
	0.151	0.058	0.024	0.100	0.411	0.090	0.219
	0.202	0.052	0.022	0.107	0.667	0.158	0.237
	0.1608	0.044	0.022	0.071	0.337	0.073	0.217
	0.020	0.079	0.034	0.124	0.442	0.129	0.292
NG	0.108	0.120	0.037	0.202	0.939	0.139	0.148
	0.150	0.107	0.036	0.204	0.742	0.175	0.236
	0.309	0.066	0.027	0.100	0.570	0.226	0.396
	0.125	0.057	0.022	0.101	0.33	0.075	0.227
	0.143	0.052	0.027	0.1	0.28	0.068	0.243
MG	0.212	0.053	0.022	0.095	0.676	0.164	0.243
	0.138	0.059	0.024	0.085	0.639	0.098	0.153
	0.182	0.056	0.026	0.1	0.657	0.132	0.201
	0.140	0.052	0.021	0.105	0.668	0.085	0.127
	0.175	0.054	0.023	0.089	0.645	0.126	0.195

Values of peak tension, time to peak tension etc. from muscle twitch experiments (direct stimulation) diabetic animals (3DP)

	F /N	t /s	$t_{1/2}$ /s	$r_{1/2}$ /s	t_d /s	TT /N s	TTI /N
DC	0.191	0.096	0.034	0.174	0.862	0.226	0.262
	0.292	0.106	0.040	0.188	0.866	0.374	0.432
	0.160	0.127	0.043	0.277	0.848	0.271	0.320
	0.090	0.084	0.035	0.143	0.874	0.094	0.108
	0.134	0.078	0.031	0.115	0.707	0.109	0.154
	0.191	0.078	0.036	0.116	0.812	0.165	0.203
	0.099	0.105	0.040	0.146	0.813	0.107	0.132
	0.095	0.095	0.041	0.138	0.741	0.095	0.128
DCR	0.177	0.118	0.045	0.216	0.800	0.253	0.316
	0.143	0.140	0.058	0.341	0.862	0.224	0.260
	0.085	0.185	0.055	0.528	1.057	0.196	0.185
	0.160	0.140	0.050	0.468	0.887	0.354	0.399
	0.185	0.078	0.030	0.095	0.587	0.153	0.261
	0.177	0.083	0.033	0.109	0.776	0.171	0.220
	0.234	0.098	0.033	0.131	0.863	0.246	0.285
	0.108	0.080	0.032	0.175	0.868	0.131	0.151
	0.225	0.098	0.040	0.116	0.790	0.221	0.280
DNN	0.043	0.088	0.037	0.110	0.552	0.029	0.053
	0.143	0.044	0.022	0.107	0.637	0.100	0.157
	0.177	0.053	0.025	0.118	0.464	0.136	0.293
	0.177	0.058	0.027	0.101	0.527	0.109	0.207
	0.185	0.076	0.033	0.137	0.658	0.173	0.263
	0.317	0.050	0.029	0.032	0.264	0.098	0.371
	0.185	0.062	0.027	0.074	0.480	0.102	0.212
	0.195	0.063	0.025	0.094	0.511	0.123	0.241
DNG	0.103	0.075	0.033	0.144	0.583	0.083	0.142
	0.090	0.059	0.027	0.135	0.806	0.094	0.117
	0.150	0.065	0.033	0.088	0.414	0.092	0.222
	0.108	0.055	0.023	0.090	0.446	0.063	0.141
	0.151	0.048	0.023	0.063	0.349	0.060	0.172
	0.063	0.064	0.028	0.105	0.341	0.040	0.117
	0.202	0.063	0.032	0.087	0.419	0.112	0.267
DMG	0.112	0.042	0.020	0.091	0.439	0.061	0.139
	0.128	0.045	0.024	0.157	0.438	0.083	0.189
	0.110	0.059	0.029	0.134	0.567	0.079	0.139
	0.111	0.062	0.028	0.144	0.692	0.106	0.153
	0.144	0.068	0.029	0.135	0.768	0.126	0.164

Appendix 17.

Values of isometric tetanic tension, reflex tension and mass of the left and right soleus muscles of non-diabetic rats (2 or 3DP)

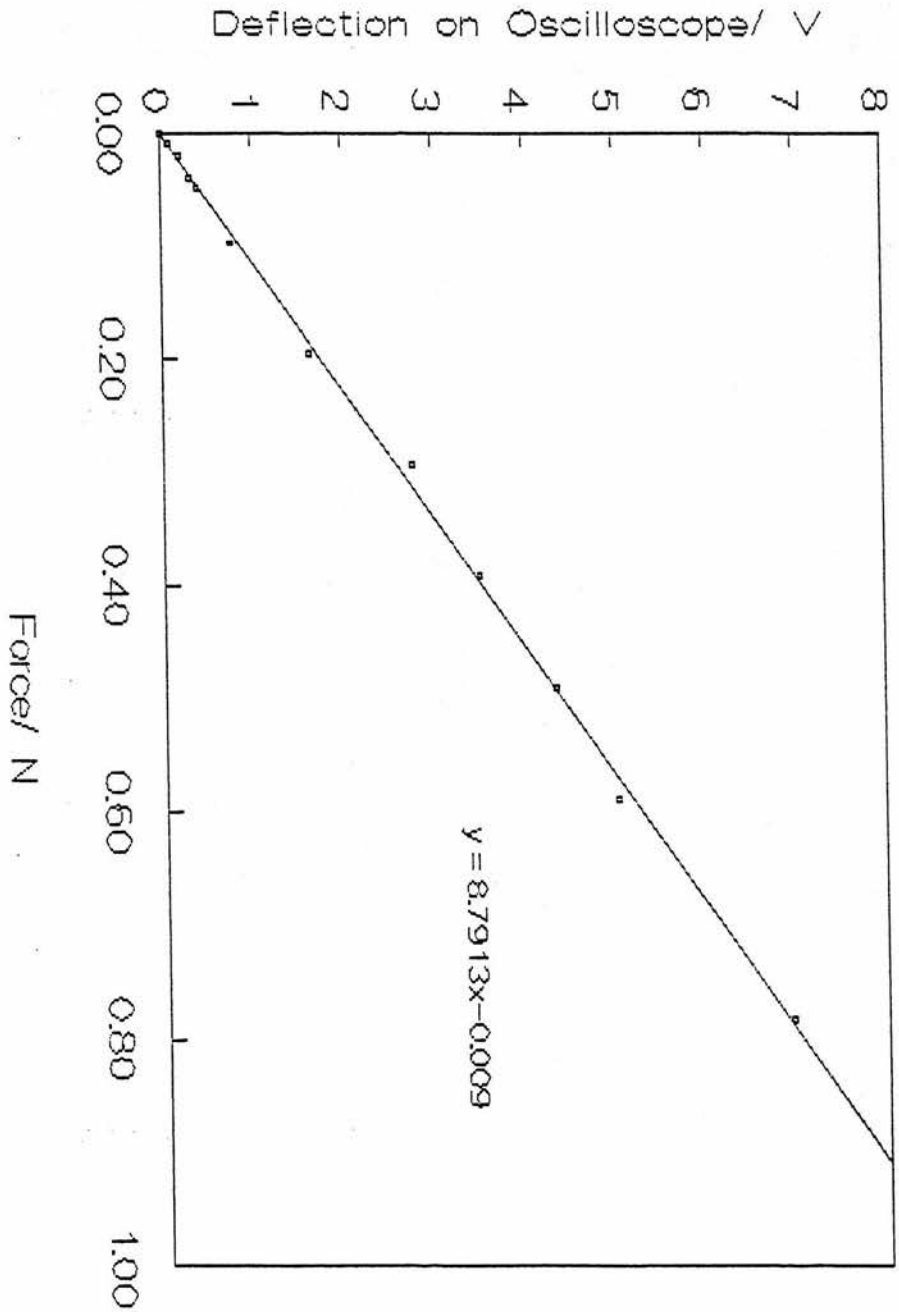
RAT GROUP	TETANIC TENSION /N	REFLEX TENSION /N	Left SOLEUS MASS /g	RightSOLEUS MASS /g
C	0.761	0.165	0.36	0.31
	0.973	0.156	0.32	0.33
	1.183	0.137	0.32	0.36
	0.928	0.179	0.31	0.37
	1.027	0.221	0.32	0.37
CR	1.517	0.165	0.34	0.37
	0.217	0.146	0.22	0.32
	0.612	0.137	0.33	0.28
	0.866	0.202	0.25	0.26
	0.692	0.163	0.32	0.24
NN	0.361		0.16	0.31
	0.309		0.21	0.34
	0.108		0.26	0.33
	0.243		0.29	0.32
	0.461		0.22	0.26
NG	0.184		0.24	0.27
	0.112		0.2	0.28
	0.381		0.35	0.43
	0.342		0.25	0.32
	0.359		0.25	0.32
MG	0.404		0.24	0.25
	0.379		0.18	0.28
	0.431		0.12	0.28
	0.225		0.25	0.32
	0.508		0.22	0.31

Values of isometric tetanic tension, reflex tension and mass of the left and right soleus muscles of diabetic rats (2 or 3DP)

RAT GROUP	TETANIC TENSION /N	REFLEX TENSION /N	Left SOLEUS MASS /g	RightSOLEUS MASS /g
DC	0.873	0.100	0.28	0.23
	0.727	0.104	0.38	0.32
	0.299	0.077	0.28	0.25
	0.376	0.084	0.29	0.29
	0.570	0.090	0.25	0.26
DCR	0.724	0.084	0.25	0.26
	0.918	0.108	0.3	0.22
	0.622	0.168	0.29	0.21
	0.115	0.147	0.24	0.23
	1.239	0.100	0.34	0.25
DNN	0.143		0.22	0.26
	0.302		0.25	0.27
	0.078		0.25	0.33
	0.333		0.29	0.32
	0.274		0.21	0.28
DNG	0.057		0.19	0.29
	0.431		0.17	0.25
	0.381		0.19	0.25
	0.112		0.19	0.26
	0.131		0.19	0.24
DMG	0.185		0.09	0.21
	0.277		0.17	0.25
	0.170		0.16	0.25
	0.148		0.15	0.26
	0.312		0.18	0.32

Appendix 18.

Calibration curve for the Lectromed 4150 force transducer.



Appendix 19.

Values of blood flux recorded from left and right sciatic nerves, before and after the administration of methoxamine, and the values of flow ratio recorded from left/operated and right/unoperated sciatic nerves of non-diabetic rats (2DP)

RAT GROUP	left FLUX /arbitrary units	right FLUX /arbitrary units	left MFLUX /arbitrary units	right MFLUX /arbitrary units	left FLOW RATIO	right FLOW RATIO
C	217.56	252.39	50.03	88.04	0.23	0.35
	199.98	94.29	49.72	42.87	0.25	0.45
	113.45	170.58	68.30	68.99	0.60	0.40
	175.34	172.39	55.69	71.24	0.32	0.41
	182.36	174.57	50.38	65.44	0.28	0.37
CR	144.86	210.78	92.65	103.00	0.64	0.49
	90.70	161.10	36.26	51.86	0.40	0.32
	80.46	134.10	46.18	59.52	0.57	0.44
	82.00	161.05	60.97	103.00	0.74	0.64
	129.84	243.82	87.58	67.84	0.67	0.28
NN	61.78	206.82	43.13	73.23	0.70	0.35
	81.97	124.60	42.74	88.80	0.52	0.71
	81.00	96.63	65.99	68.53	0.81	0.71
	76.98	99.80	51.10	52.93	0.66	0.53
	44.02	88.08	38.34	78.36	0.87	0.89
NG	100.36	198.64	41.48	93.98	0.41	0.47
	60.12	100.54	38.58	45.28	0.64	0.45
	117.90	247.85	42.18	54.26	0.36	0.22
	132.60	140.9	50.20	48.00	0.38	0.34
	138.90	139.2	42.40	53.00	0.31	0.38
MG	56.94	205.46	48.57	68.30	0.85	0.33
	79.15	195.05	54.23	63.15	0.69	0.32
	86.60	171.42	42.48	81.38	0.49	0.47
	85.00	195.30	47.00	70.80	0.55	0.36
	73.20	187.96	46.20	69.20	0.63	0.37

Values of blood flux recorded from left and right sciatic nerves, before and after the administration of methoxamine, and the values of flow ratio recorded from left/operated and right/unoperated sciatic nerves of diabetic rats (2DP)

RAT GROUP	left FLUX /arbitrary units	right FLUX /arbitrary units	left MFLUX /arbitrary units	right MFLUX /arbitrary units	left FLOW RATIO	right FLOW RATIO
DC	130.00	94.86	53.26	25.92	0.41	0.27
	108.45	107.75	59.88	68.83	0.55	0.64
	139.72	205.38	41.17	58.10	0.29	0.28
	163.81	149.57	45.70	49.16	0.28	0.33
	135.50	139.45	43.21	57.39	0.32	0.41
DCR	49.10	181.25	48.05	78.40	0.98	0.43
	115.15	192.57	78.23	49.12	0.68	0.26
	75.68	90.63	37.63	79.88	0.50	0.88
	86.73	130.02	38.63	55.42	0.45	0.43
	79.64	90.66	33.97	73.47	0.43	0.81
DNN	87.00	197.24	51.16	82.48	0.59	0.42
	96.44	144.74	34.66	109.41	0.36	0.76
	117.26	193.23	47.56	63.44	0.41	0.33
	135.46	155.13	47.62	88.22	0.35	0.57
	107.38	134.23	41.50	56.28	0.39	0.42
DNG	88.00	188.24	29.04	60.13	0.33	0.32
	106.96	120.28	42.71	52.17	0.40	0.43
	114.42	217.72	60.43	91.13	0.53	0.42
	112.30	178.00	44.00	73.50	0.39	0.41
	105.20	170.50	41.20	65.70	0.39	0.39
DMG	44.6	103.05	27.49	49.34	0.62	0.48
	41.74	164.49	25.53	49.96	0.61	0.30
	42.20	132.67	26.80	42.90	0.64	0.32
	39.20	133.12	29.76	98.11	0.76	0.74
	94.50	267.49	55.64	74.18	0.59	0.28